From The Department of Clinical Science and Education,
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PEDiatric Inflammatory BOWel Disease:
CLINICAL AND IMMUNOLOGICAL ASPECTS ON REMISSION TREATMENT

Helena J. Rolandsdotter

Stockholm 2017
PEDIATRIC INFLAMMATORY BOWEL DISEASE: CLINICAL AND IMMUNOLOGICAL ASPECTS ON REMISSION TREATMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Department of Medicine, Solna
To all kids with inflammatory bowel disease, and especially to those that made these studies possible by so graciously giving your time, your blood and your guts - rarely with a complaint, and almost always with a smile.

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ABSTRACT

Background
Inflammatory bowel disease (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), are lifelong conditions characterized by abdominal pain, bloody diarrhea and fatigue. The incidence and prevalence are increasing worldwide, with approximately 10-20% of all IBD cases diagnosed during childhood. The etiology is considered multifactorial but is not completely understood. However, genetic susceptibility, environmental factors and disturbed immunological function appear to contribute to the development of IBD. The treatments of pediatric CD and UC are only in part the same. Unfortunately, we still frequently use high dosage of corticosteroids, and we do not practice personalized medicine because of a lack of knowledge about which treatment best suits the individual patient. In our ambition to better understand the pathophysiology of IBD and the mode of action of established therapies, as well as to determine new therapy strategies, we studied the clinical effect of Infliximab (IFX) in children on maintenance treatment and the therapeutic effect of granulocyte and monocyte apheresis (GMA) in children with newly onset IBD. In addition, children with CD were treated with exclusive enteral nutrition (EEN) as induction of remission therapy. Finally, we studied the immunological profile in blood at onset and in intestinal mucosa at onset and after GMA and EEN treatment.

Methods and results
We investigated the association between s-IFX trough levels and antibodies to IFX (measured with ELISA, enzyme-linked immunosorbent assay) to clinical indices and CRP, ESR, albumin and F-calprotectin in 45 children on maintenance IFX treatment. The mean s-IFX trough levels were significantly higher during remission than in active disease, correlating to the clinical indices, ESR, CRP and albumin. The development of antibodies to IFX strongly correlated to undetectable s-IFX and active disease (Paper I). In paper II (pilot study), 12 children with newly IBD colitis received 10 sessions with GMA together with a low to moderate dose of mesalazine as induction of remission. A control colonoscopy (CC) was performed 12 to 16 weeks post-treatment, in which the endoscopic Mayo score showed significant improvement in 9/12 children (8/12 were in clinical remission). In seven of these children (paper V), the expressions of 14 cytokines were investigated (by real time polymerase chain reaction, PCR) in the intestinal mucosa at onset and after the combination therapy of GMA and mesalazine. Significant decreases were seen in CSF-2, TNF-α, IL-23α, IL-1β, IL-36γ, IL-10 and TGFβ1 after treatment while significant decreases were observed in the clinical index and Mayo
endoscopic score. Compared with the IBD patients, significantly lower levels of IL-12β and IL-23α were found in the six non-IBD controls at onset. In paper III, we characterized the chemokine receptor landscape in 45 children (UC: n=16, CD: n=12 and healthy controls: n=17) using flow cytometry. By defining a diagnostic algorithm based on these markers, we could distinguish UC from CD in >92% of the studied children with newly onset IBD. In paper IV, 12 children with newly onset IBD were treated with six weeks of EEN as induction of remission therapy. Eleven of the 12 patients successfully completed the treatment. A CC after completion of EEN showed significant decreases in endoscopic scoring (SES-CD) and 83% of the patients were in clinical remission. Additionally, in six of the children mucosal cytokines were measured by real time PCR at diagnosis and at CC. An overall decrease (though not statistically significant) in pro-inflammatory cytokines, as well as both decreases and increases in the regulatory cytokines, were seen after EEN therapy.

Conclusions
We conclude that an active approach is needed in the care of children with IBD to achieve and maintain remission. Our findings reveal that the children on IFX maintenance treatment were only in remission in 28% of the visits. The combination of GMA and mesalazine was found to be a safe and effective treatment in children with newly onset IBD. It seems plausible to speculate that the decreases in mucosal cytokines after the induction of remission may explain the good clinical result. Moreover, a change in the mucosal cytokine profile after induction of remission with EEN was observed. By investigating the chemokine receptors, we found a possible prognostic IBD marker, and by analyzing the cytokine profiles in mucosal biopsies, we have extended the knowledge of immunological phenotypes in children with IBD.

Suggestions for the future
Corticosteroid-free treatment alternatives must be explored and those currently in use must be optimized. To conclude, more and bigger studies are needed to explore the pathogenesis of IBD to determine new treatment alternatives.

II. Rolandsdotter H, Eberhardson M, Fagerberg UL, Finkel Y.

III. Linton L, Rolandsdotter H, Hyllienmark M, Finkel Y, Winqvist O, Eberhardson M.
Chemokine receptor on blood leukocytes; a potential diagnostic tool in children with inflammatory bowel disease.
Submitted

IV. Rolandsdotter H, Videsäter-Jönsson K, Fagerberg UL, Eberhardson M, Finkel Y.
Exclusive enteral nutrition: clinical effects and changes in mucosal cytokine profile in children with first onset inflammatory bowel disease
In manuscript

V. Rolandsdotter H, Videsäter-Jönsson K, Fagerberg UL, Eberhardson M, Finkel Y.
EEN for induction of remission and changes in mucosal cytokine profiles

IFX trough levels and antibodies to IFX

Colonic mucosal cytokine profiles and clinical outcome

Chemokine receptors on blood leukocytes as a prognostic marker for CD and UC

EEN for induction of remission and changes in mucosal cytokine profiles

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>5-ASA</td>
<td>5-Aminosalicylates</td>
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<tr>
<td>ADA</td>
<td>Adacolumn®</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>ATI</td>
<td>Antibodies toward infliximab</td>
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<td>AZA</td>
<td>Azathioprine</td>
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<td>CC</td>
<td>Control colonoscopy</td>
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<td>CCR</td>
<td>Chemokine receptor</td>
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<tr>
<td>CD</td>
<td>Crohn’s disease</td>
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<td>CDAI</td>
<td>Crohn’s disease activity index</td>
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<td>CDEIS</td>
<td>Crohn’s disease endoscopic index of severity</td>
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<td>CMCP</td>
<td>Colonic mucosal cytokine pattern</td>
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<td>CRP</td>
<td>C-Reactive protein</td>
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<td>CS</td>
<td>Corticosteroids</td>
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<td>DC</td>
<td>Dendritic cells</td>
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<td>EEN</td>
<td>Exclusive enteral nutrition</td>
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<td>ESPGHAN</td>
<td>European Society of Gastroenterology, Hepatology Nutrition</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<td>F</td>
<td>Fecal</td>
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<tr>
<td>FCP</td>
<td>Fecal calprotectin</td>
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<td>GI</td>
<td>Gastrointestinal</td>
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<td>GMA</td>
<td>Granulocyte monocyte apheresis</td>
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<td>GSF</td>
<td>Granulocyte colony stimulating factor</td>
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<td>IBD</td>
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<td>IBD unclassified</td>
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<td>IEC</td>
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<td>IL</td>
<td>Interlukein</td>
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<td>LOR</td>
<td>Loss of response</td>
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<tr>
<td>NK</td>
<td>Natural killer cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCDAI</td>
<td>Pediatric Crohn’s Disease Activity Index</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>PSC</td>
<td>Primary sclerosing cholangitis</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>TDM</td>
<td>Therapeutic drug monitoring</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TGF</td>
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INTRODUCTION

My research field and clinical work focus on pediatric Crohn’s disease (CD) and ulcerative colitis (UC). CD and UC are the most common types of inflammatory bowel disease (IBD), and a sharp increase in the incidence have been seen during the last decades. IBD seems to have followed by humanity: in the ancient Chinese Yellow Emperor’s canon of internal medicine (722-721 BC), an illness is described as resembling UC with abdominal pain, diarrhea and rectal bleeding. In 1859, sir Samuel Wilks, an English physician, described IBD more than 70 years before Dr Burrill Crohn published a paper on “regional ileitis” in 1932.1-2.

Childhood is a comparatively short period in an individual’s life cycle. During this extremely critical period, personality and social skills develop, school and avocations take time and energy and physiological and physical development start to mature. Unfortunately, this is not always easy for children with IBD. The course of the disease is quite unpredictable, with frequent flares and sometimes it appears the bathroom is visited more often than school, activities and friends.

Now, we do not have the required knowledge to predict the prognosis of the patients. More knowledge about the pathogenesis and how different treatments work are desirable. A more personal designed medication is needed but currently our knowledge about which treatment suits which patient is incomplete. When we started this research project, I thought it was all about finding new strategies and results that would be applied to benefit patients in the treatment or diagnosis of IBD.

This goal is still important, but I have realized that going through the PhD process is also a personal journey in which I have learned a lot about myself and others.

Many things did not turn out exactly as planned. Sometimes the level of frustration reached quite high levels, and more than once I asked myself, why did I take this road. On the other hand, a successful research result can bring about incredible satisfaction and joy. Research itself is a dynamic process and many researchers are enthusiastic and dynamic people. I would like to share some of my reflections and experiences (both of the results and more practical matters) that I have made during this long and inspiring journey, which you will find in the reflection squares in the last part (discussion).

Helena Rolandsdotter
Stockholm 2017
1 BACKGROUND

1.1 DEFINITIONS

IBD is a group of inflammatory diseases of the colon and upper intestinal tract characterized by chronic intestinal inflammation. The disease is normally divided into the following conditions:

CD: This condition may affect any part of the gastrointestinal (GI) tract from mouth to anus. The inflammation is transmural (extending through the intestinal wall) and often discontinuous and segmental. Extraintestinal manifestations involving joints, eyes, skin and liver may occur as well as intestinal complications such as fistulas, perianal abscesses and stenosis.

UC: This disorder affects only the rectum and colon. The inflammation involves the mucosa (the lining coat of the colon) and is continuous, but the extension differs from distinct proctitis to pan-colitis. It may also include extraintestinal symptoms from joint, eyes, skin and liver.

IBD unclassified (IBDU): A distinction cannot be made between CD or UC despite extensive investigations with upper and lower endoscopy, histopathology and examination of the ileum.

CD and UC share several common symptoms, such as diarrhea, often with blood, abdominal pain and cramping, reduced appetite and nausea, as well as an overall impaired general condition. In CD, mouth ulcers, concomitant fever and fistulas may present. Growth impairment is more usual in children with CD (reported in 10-20% at diagnosis) than in children with UC\(^3,6\). Gross bleeding and anemia at diagnosis are more often seen in UC patients than in patients with CD. Patients with IBD may also entail considerable mental suffering and anxiety compared with people without IBD\(^3,1\).

1.2 EPIDEMIOLOGY

The incidence and prevalence of IBD have steadily increased worldwide and has become a global emergence disease in the past decades, especially in developed countries, with the disease affecting about 1 in 200 people\(^6\). In a report on IBD incidence in Denmark between 1980 and 2013, the incidence of CD increased from 5.2 per 100,000 to 9.1 per 100,000 while the incidence of UC increased from 10.7 per 100,000 to 18.6 per 100,000. The increase in CD was greatest in patients aged <15 years; the increase in UC was seen in patients >15 years of age.

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age. Furthermore, the incidence rates for women were significantly higher in both UC and CD than for men. Today, the overall prevalence of IBD in Sweden is approximately 0.65%.

Approximately 10-20% of all IBD cases are diagnosed during childhood and onset is most common in adolescence to young adulthood, with a peak at 15-29 years. However, several studies have reported a rise in early onset IBD (diagnosis at age <10 years). The incidence of pediatric IBD has increased sharply in recent decades, as is true for the whole Northern hemisphere.

In Sweden, prevalence of pediatric IBD in 2010 was 75/100,000 (0.75 % of all children in Sweden in 2010). Figure 1 depicts the incidence rate from 2003 to 2013 by type of IBD, sex and age of onset.

1.3 PATHOPHYSIOLOGY

The causes of IBD are not clear and considered multifactorial, which is because of inflammatory responses and genetic factors. In short, genetic susceptibility, environmental factors and disturbed immunological function appear to contribute to the development of IBD.

Figure 1: Incidence rate of pediatric IBD per 100,000 in Sweden 2003-2013, by type of IBD, sex and age of onset.

Everhov et al. AP&T, 2017
1.3.1.1 Genetics in IBD

201 susceptibility loci for IBD, mostly shared between CD and UC have hitherto been discovered. However, many IBD loci are also implicated in other autoimmune-mediated disorders, most notably with ankylosing spondylitis and psoriasis. Many of the involved genes regulate the ability of intestinal intraepithelial cells (IECs) to insulate themselves from direct microbial contact, handle the stress of metabolic and environmental factors and encode for proteins involved in autophagy. A strong genetic factor in the development of IBD has been refuted. First reported in twin-studies, the CD concordance rates were between 33 and 50% in monozygotic twins and between 3 and 10% in dizygotic twins. Heredity seems to play a larger part in CD than in UC. A first-degree relative to a patient with IBD has a tenfold increased risk of developing the same disease as the relative when compared with the general population. In the largest genotype association study to date, three loci (NOD2, MHC, MST1 3p21) represent different sub-phenotypes that could be characterized primarily by the disease location: ileal CD, colonic CD and UC. The authors suggest that this nomenclature should be used instead of CD and UC as currently defined.

1.3.1.2 Environmental factors

Smoking and GI infections have the strongest relationship to environmental factors that may trigger the onset of IBD. In CD, early tobacco use increases the risk for disease development; in UC, current smoking protects against the IBD. Both onset and disease activity may be linked to smoking cessation in UC patients. Some GI infections seem to trigger IBD onset: both UC and CD occur after previous infections with Salmonella spp, Mycobacterium avium, Shigella spp and Campylobacter spp, suggesting there is an alteration in the gut flora that triggers the start of a chronic inflammation process, and the reverse; some report have shown that helminth infection during childhood seems to protect against IBD. Infections with Clostridium difficile, a gram-positive, anaerobic, spore-forming bacillus, may also worsen the course of IBD.

Theories of other environmental factors that may influence the increasing incidence of IBD have been suggested in terms of a higher living standard, including better hygiene and sanitation, smaller families, less women breastfeeding, consumption of a western diet and...
A difficult complication of pediatric IBD is growth failure. All these factors may contribute but none have shown, either alone or in combination, any convincing association with the augmented incidence of IBD.

1.3.1.3 Immunological dysfunction (short version)
Comprehensive knowledge of the immune system plays a crucial role in understanding the pathophysiology of IBD, including knowledge of the intestinal barrier, the innate immune system and the adaptive immune system. The first line of defense is the mucosal barrier, a thin sheet of mucus secreted mainly by goblet cells, which also synthesize antimicrobial peptides. In both UC and CD, this barrier is dysfunctional and shows increased permeability due to T cell-mediated disruption of the tight junction protein and enteric neuron dysfunction. The innate immune responses include monocytes, macrophages and dendritic cells (DCs) that increase in correlation with disease activity. The innate immune cells present antigens to T and B cells that take part in the adaptive immune system and disturb the delicate balance between regulatory T cells and helper T cells

1.4 PEDIATRIC IBD
Children and youth often have more severe and more extensive disease than adults, with vast involvement and rapid early progression. The CD phenotype shows less isolated ileal disease compared with adults and is characterized by a pan-enteric phenotype. Eighty-two percent of children with UC have extensive disease as compared with 48 percent of the adult population.

A difficult complication of pediatric IBD is growth failure, which is more severe in CD than in UC. Several factors contribute, such as loss of appetite, protein loss due to mucosal inflammation, malabsorption due to an inflamed mucosa, ongoing inflammation followed by an increased nutritional need, and corticosteroid (CS) treatment that interferes with growth. Studies have shown an association between levels of circulating inflammatory cytokines (interleukin 6 [IL-6] and tumor necrosis factor alpha [TNF-α]) and reduced growth velocity and impaired body composition.

1.5 DIAGNOSTIC WORK-UP
A complete medical history, physical examination, laboratory testing that includes full blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), albumin, liver function

6
tests, transglutaminase antibody, fecal calprotectin and stool cultures to exclude infectious diarrhea are needed to diagnose a child with suspect IBD. Additionally, all children with suspected IBD should undergo a gastro-duodenoscopy and ileocolonoscopy as well as small bowel imaging with magnetic resonance imaging (MRI) or ultrasound, according to the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) revised Porto criteria for the diagnosis of pediatric IBD from 2014. Thus, the diagnosis is made based on history, physical examination, biochemistry and macroscopic and histological findings from the gastro-colonoscopy.

1.5.1 Endoscopy and histology in IBD

All endoscopic procedures during childhood are done in general anesthesia. An upper endoscopy and ileocolonoscopy with multiple biopsies from the esophagus, corpus, antrum and the proximal and descending duodenum, ileum, caecum, ascending, transverse, descending and sigmoid colon and rectum are performed according to the European Crohn's and Colitis Organization (ECCO)-ESPGHAN guidelines for newly onset IBD.

CD and UC may demonstrate different appearances of gastro-colonoscopy: patients with CD typically show segmental and deeper intestinal inflammation that can involve any part of the GI tract. Children with UC commonly show a superficial and continuous inflammation. Table 1 shows endoscopic characteristics and Table 2 histologic characteristics of CD and UC.

Table 1. Endoscopic characteristics of CD and UC

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</tr>
<tr>
<td>Submucosal or transmural involvement</td>
<td>Mucosal involvement</td>
</tr>
<tr>
<td>Ulcers, crypt distortion</td>
<td>Crypt distortion</td>
</tr>
<tr>
<td>Crypt abscesses</td>
<td>Crypt abscesses</td>
</tr>
<tr>
<td>Granulomas</td>
<td>Goblet cell depletion</td>
</tr>
<tr>
<td>Focal changes (within biopsy)</td>
<td>Continuous distribution</td>
</tr>
<tr>
<td>Patchy distribution (between biopsies)</td>
<td>Patchy distribution (between biopsies)</td>
</tr>
</tbody>
</table>

1.6 SCORING SYSTEMS

Several scoring and classification systems have been developed for clinical use and research purposes. In clinical practice, scoring systems make it easier for the physician to evaluate the patient and in research different study results can be compared. Although, having a wide variety of scoring systems may be confusing. Not surprisingly, not all scoring systems are in use today in clinical practice. Pediatric gastroenterologists often use different scoring systems than gastroenterologists working exclusively with adult patients.

1.6.1 Clinical scoring

The Pediatric CD Activity Index (PCDAI) is widely used and has been found to be a reliable tool for intervention trials in CD. The index describes four general dimensions during the last week: history, physical examination, growth parameters and common laboratory tests. Disease activity is as follows: <10 point = remission, 10-27.5 = mild disease, >27.5-37.5 = moderate disease and >37.5-100 = severe disease. A PCDAI decrease of ≥12.5 points reflects a clinically significant response to treatment. Newer, shorter versions of the PCDAI have been constructed (weighted PCDAI, short PCDAI, abbreviated PCDAI and modified PCDAI). These newer versions are considered more feasible to use than the original version.

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The Pediatric UC Activity Index (PUCAI) is widely used to score patients in the everyday clinical setting, as well as for research purposes. The answers reflect the status of the patient during the last two days and encompass abdominal pain, rectal bleeding, stool consistency, number of stools per 24 hours, nocturnal stools and activity level. A PUCAI <10 is interpreted as remission, 10-34 as mild disease, ≥35-64 as moderate disease and ≥65-85 as severe disease. A significant improvement is construed as a change in PUCAI of ≥35, moderate improvement ≥20-34 and small improvement ≥10-19.

Other clinical CD scores currently used in adult gastroenterology are the CD Activity Index (CDAI), and the Harvey and Bradshaw’s Activity Index (HBI), which is a simpler version of the CDAI. In UC, the Modified Truelove and Witt’s Severity Index and the Mayo score have been frequently used (Mayo including endoscopic findings and partial Mayo without endoscopic findings).

1.6.2 Classification
The most used phenotype classification in pediatric IBD, the Paris classification, is a development of the Montreal classification that had several weaknesses with respect to classification of children (change in disease location and behavior over time). It gives possibilities to find a genotype-phenotype association, which is of great value in research, but also makes it easier to follow the course of the patients in a structured manner (Table 3).
Table 3. The Paris classification of pediatric IBD describes the phenotype of both UC and CD.

<table>
<thead>
<tr>
<th>Location</th>
<th>Ulcerative proctitis</th>
<th>Left-sided UC, distal to splenic flexure</th>
<th>Extensive, hepatic flexure distally</th>
<th>Pancolitis, proximal to hepatic flexure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severity 0</td>
<td>Never severe</td>
<td>Defined as PUCAI ≥65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severity 1</td>
<td>Ever severe</td>
<td>Defined as PUCAI ≥65</td>
<td></td>
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**L4a: upper disease proximal to ligament of Treitz**

<table>
<thead>
<tr>
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</tbody>
</table>

**L4b: upper disease distal to ligament of Treitz**

**Cerumen disease**

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>A1: 0–10 y</th>
<th>A2: 11–40 y</th>
<th>A3: &gt;40 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>L1: distal 1/3 ileum</td>
<td>L2: colonic</td>
<td>L3: ileocolonic</td>
</tr>
</tbody>
</table>

**Behavior**

| B1: non-structuring, non-penetrating | B2: stricturing | B3: penetrating |

**Growth**

| G1: growth delay | G0: no evidence of growth delay |

**Personal disease**

| B: B1B2B3: both stricturing and penetrating disease |

In the Paris classification system, L4 and L4a/L4b may coexist with L1, L2, L3.

**L4a: upper disease proximal to ligament of Treitz, L4b: upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum.

1.6.3 Endoscopic scoring

Why is a reliable endoscopic scoring system so important to achieve? Professor Marcus Neurath elegantly summarized this in the following comment:

The structural basis of mucosal healing is an intact barrier function of the gut epithelium that prevents translocation of commensal bacteria into the mucosa and submucosa with subsequent immune cell activation. Thus, mucosal healing should be considered as an initial event in the suppression of inflammation of deeper layers of the bowel wall, rather than as a sign of complete healing of gut inflammation.

1.6.3.1 Crohn’s disease: endoscopic indices

For CD, there are mainly two endoscopic indices in use. One index is the CD Endoscopic Index of Severity (CDEIS). Deep and superficial ulcerations for five segments and the presence or

1.6.3 Endoscopic scoring

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1.6.3.1 Crohn’s disease: endoscopic indices

For CD, there are mainly two endoscopic indices in use. One index is the CD Endoscopic Index of Severity (CDEIS). Deep and superficial ulcerations for five segments and the presence or
absence of stenosis (ulcerated and non-ulcerated stenosis) are obtained to produce this validated scoring\(^5\). This scoring correlates well with the clinical scoring CDAI but is known to be complicated to use. The other index, the Simple Endoscopic Score (SES-CD), is also frequently used and correlates well with the CDEIS. It scores ulcer size, ulcerated surface, affected surface and luminal narrowing in five segments. The CD-SES is easier to use than the CDEIS. Regrettably, no endoscopic indices measure upper inflammation, which is not unusual in pediatric CD.

1.6.3.2 Ulcerative colitis: endoscopic indices

The modified Baron score, developed from the original Baron score (1964), describes the mucosa as normal, mild, moderate and severe\(^5\). The newer Ulcerative Colitis Endoscopic Index of Severity (UCEIS) is a validated scoring system comprising three components (vascular pattern, bleeding, erosions and ulcers), each with precise definitions and three or four levels of severity, yielding a 9-point scale\(^5\).

The Mayo endoscopic score is a widely used three-point scale that has not yet been validated (Table 4)\(^5\).

<table>
<thead>
<tr>
<th>Endoscopic Mayo score</th>
<th>Endoscopic findings</th>
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<tr>
<td>Score 0 = remission</td>
<td>Normal mucosa</td>
</tr>
<tr>
<td>Score 1 = mild disease</td>
<td>Erythema, decreased vascular pattern, mild friability</td>
</tr>
<tr>
<td>Score 2 = moderate disease</td>
<td>Marked erythema, lack of vascular pattern, friability and erosions</td>
</tr>
<tr>
<td>Score 3 = severe disease</td>
<td>Spontaneous bleeding, ulceration</td>
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Table 4. Mayo ulcerative colitis endoscopic score.

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</table>

Table 4. Mayo ulcerative colitis endoscopic score.
1.6.4 Histologic scoring

In a recent meta-analysis of 15 studies (n=1573 patients), the predictive power of histology was analyzed. This report concluded that histologic remission correlates to less flares and that histologic remission was superior as a predictor of outcomes compared with endoscopic and clinical remission57.

Nevertheless, another recent meta-analysis that evaluated the development and operating characteristics of histologic disease activity indices of UC found that none of the currently available histologic scoring indices have been fully validated58. The same condition prevails for CD, i.e. no fully validated histological scoring index is available. In a systemic review of evaluating available histological disease indices, the Nainia and Cortina Score was assessed for feasibility and found to be easily administered57,59.

That active disease reflects neutrophils in the crypt epithelium and crypt lumen (cryptitis and crypt abscesses) seems to be a generally agreed upon marker of disease activity, whereas other histological features are more variable across studies.80 Mild inflammation (close to remission) is characterized by the lack neutrophils but various degrees of chronic inflammation may persist.

The Geboes score is a widely used instrument to measure disease activity81. This histopathologic scoring system uses a six-point grading system: architectural changes, chronic inflammatory infiltrate, lamina propria neutrophils and eosinophils, neutrophils in epithelium, crypt destruction and erosions or ulcerations. Each grade of the scoring system is divided into four subcategories. A simplified Geboes score has been developed because the original Geboes is somewhat complicated to use. Both the simplified and the original Geboes instruments have not been fully evaluated80.
Geboes score: different grades used to evaluate disease severity in UC.

<table>
<thead>
<tr>
<th>Grade 0: Structural (architectural change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 No abnormality</td>
</tr>
<tr>
<td>0.3 Severe diffuse or multifocal abnormalities</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade 1: Chronic inflammatory infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 No increase</td>
</tr>
<tr>
<td>1.3 Marked increase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade 2: Lamina propria neutrophils and eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A Eosinophils</td>
</tr>
<tr>
<td>2A.0 None</td>
</tr>
<tr>
<td>2A.3 Marked increase</td>
</tr>
<tr>
<td>2B Neutrophils</td>
</tr>
<tr>
<td>2B.0 None</td>
</tr>
<tr>
<td>2B.3 Marked increase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade 3: Neutrophils in epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 None</td>
</tr>
<tr>
<td>3.3 ≥ 50% crypts involved</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade 4: Crypt destruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 None</td>
</tr>
<tr>
<td>4.3 Unequivocal crypt destruction</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade 5: Erosion or ulceration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 No erosion, ulceration, or granulation tissue</td>
</tr>
<tr>
<td>5.2 Probable erosion/focally stripped</td>
</tr>
<tr>
<td>5.3 Unequivocal erosion</td>
</tr>
<tr>
<td>5.4 Ulcer or granulation tissue</td>
</tr>
</tbody>
</table>

Position of the neutrophils between the epithelial cells scored separately in grade 3

<table>
<thead>
<tr>
<th>Surface epithelium</th>
<th>Crypt epithelium</th>
<th>Crypt abscences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0 X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0 X X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.7 PEDIATRIC IBD TREATMENTS

The main treatment goal for CD and UC is mucosal healing, which is normally followed by optimized growth, relief of symptoms and improved quality of life. Treatment is divided into remission and maintenance therapy: remission therapy aims to decrease the inflammatory
burden while the aim of maintenance treatment is to prolong treatment response and delay the
time to relapse.

Today, we do not practice personalized treatment regimes because of a lack of knowledge about
which treatment best suits the individual patient. Instead, we most often employ the step-up
model of treatment for CD and UC, starting with a treatment that has been in use for a long time
(most often 5-Aminosalicylates [5-ASA], CSs and EEN) and proceed with other more exclusive
and expensive treatments when there is a lack or loss of response.

The strategy of starting with more exclusive treatments as induction of remission (i.e. a top-
down approach) has been debated over the past decade. According to a new pediatric
American report, early anti-TNF treatment seemed to increase over time and was related to
lower rates of CS use compared with the conventional approach.

Severe side effects are a difficult clinical problem for most medical treatments. This problem is
particularly true for CSs. Thus, efficacy of treatment must always be weighed against harmful
side effects. IBD in the pediatric population must be closely monitored by measuring f-
calprotectin, blood chemistry and validated scoring indexes (e.g., the PCDAI and PUCAI).
Surgery is often the treatment of last resort when there is no clinical response to other
treatments, including intravenous CSs.

1.7.1 Aminosalicylates

The 5-ASAs are released in different locations throughout the intestine: controlled release
(Pentasa), pH dependent release (either pH 6 or 7) delivered distal of the distal ileum (Salofalk,
Mesoril or Asacol) and azo-compounds that are delivered in the colon by bacterial cleavage
(sulfasalazine). The mode of action of 5-ASA is not fully understood, but appears to act
locally on colonic mucosa by activating a key receptor referred to as the Peroxisome
proliferator-activated receptor (PPAR)γ (a member of the steroid nuclear receptors), which is
involved in apoptosis, cell proliferation, cytokine production and anti-tumorigenic effects.
5-ASA given orally is a cornerstone in the treatment of mild extensive UC and is considered a
safe treatment modality. It may also be given to patients with mild CD colitis as a first-line
treatment but is not useful in upper and ileal CD. Isolated proctitis/left-sided colitis can be
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regimes are equally effective as short-term treatment of active UC in children.
1.7.2 Corticosteroids

CSs (also called glucocorticoids, corticosteroids or steroids) affect the innate and adaptive immune systems. The anti-inflammatory effect is mediated by four different main modes of action\textsuperscript{84}. Through a mechanism termed transactivation, a cytoplasmic intracellular receptor for glucocorticoids affect a glucocorticoid-responsive element in the DNA, which induces the production of anti-inflammatory proteins\textsuperscript{89}. Transactivation is also responsible to the CS side-effects by an induction of gluconeogenic enzymes\textsuperscript{84}. The receptor also binds to glucocorticoids-responsive elements in the DNA and thereby inhibiting gene expression of IL-1 and IL-2. Transrepression involves the binding of the receptor complex to pro-inflammatory transcription factors in the genome, thus preventing the transcription of activator protein 1 and NF-κB\textsuperscript{90}, and further, the receptor complex competitively inhibits co-activators in the nucleus followed by reduced expression of cytokines, like TNF-α and IFN-γ\textsuperscript{84}.

CSs are recommended for children with moderate to severe UC with systemic symptoms\textsuperscript{81} and up to 80% of the children with UC are treated with CS, mainly within 3 months of diagnosis. The short-term response rate varies from 50 to 90\%\textsuperscript{67,81,82}. Nevertheless, the correlation between clinical remission and endoscopic remission after cortisone treatment is low\textsuperscript{73}. In children with CD, EEN is the first-line treatment, but CS therapy may be a viable alternative\textsuperscript{84}. CSs could be taken orally, as suppository or enemas, or intravenously. Indeed, a corticosteroid-sparing treatment is desired for children, largely because of the diversity of side effects: sleeping problems, euphoria, anxiety, nausea, excessive appetite followed by weight gain, stomach pain, acne, striae and growth impairment\textsuperscript{84}. If CSs must be used, the treatment should be tapered as soon as possible because of the risk of these side effects\textsuperscript{81,82}. A difficult clinical problem is steroid dependency (defined as remission with CS but recurrence of symptoms when the dose is lowered, or if steroids cannot be stopped within 14-16 weeks). Indeed, the benefit of finding more CS free remission treatments for children and adolescents is of great importance.

1.7.3 Exclusive enteral nutrition

Children and youth with CD often present with growth impairment, both at onset and later in the disease course, which is due to GI inflammation, malnutrition and the use of CSs\textsuperscript{75}. Therefore, it is important to consider other treatment modalities. In fact, the current standard treatment of induction of remission in pediatric patients with CD in Europe is 6-8 weeks with exclusive enteral nutrition (EEN)\textsuperscript{84}. EEN consists of liquid formulas, either elemental...
(formulations of amino acids), semi-elemental (formulations of amino acids and oligopeptides) or polymeric (whole protein formulas). This treatment has an overall clinical remission rate of 73-80%; it effectively induces mucosal healing (MH) and is superior to CSs\textsuperscript{41-43}. It should be given to all children with luminal disease, including those with colonic involvement. EEN can be given orally or by a feeding tube\textsuperscript{44}. The mechanism of action of EEN has not been elucidated, although different underlying mechanisms have been proposed, including correction of intestinal permeability, diminution of intestinal synthesis of inflammatory mediators via reduction in dietary fat, elimination of dietary antigen uptake and provision of important micronutrients to the diseased intestine. However, no strong evidence has been provided for any of these explanations. Children in remission following EEN have shown altered fecal microflora during and after EEN, suggesting that the change in gut microflora may induce remission\textsuperscript{80-81}. One overriding benefit of EEN is that the often-malnourished child at CD onset not only obtains remission but also a positive nutritional status. Consequently, this treatment is favored before others. Body composition seems to improve with EEN, which is known to promote anabolism by reducing proteolysis and increasing protein synthesis\textsuperscript{82}. However, many patients find it difficult to subsist on only a liquid diet without any other food for 6-8 weeks. This issue constitutes one of the main drawbacks of ENN treatment. A strict schedule must be drawn up by a dietician, and the physician and dietician must engage with the patient collaboratively for the child to continue the treatment.

1.7.4 Thiopurines - Azathioprine and 6-Mercaptopurine

Thiopurines exert their anti-inflammatory effect through the formation of the active metabolite 6-thioguanin-triphosphate (6-TGTP), which causes apoptosis of activated T-lymphocytes when administered in low dosage\textsuperscript{83}. Small, retrospective studies\textsuperscript{84, 85} have shown that Azathioprine (AZAs) are effective and associated with prolonged maintenance of remission in CD and decreased rates of hospitalization, CS use and need of surgery. AZA and 6-MP are also used as a maintenance treatment in children with UC\textsuperscript{86}. However, the results of different reports concerning the efficacy of thiopurines are contradictory. In a review that consisted of 13 randomized trials with 1211 adult CD patients, AZA and 6-MP were found to be no more effective than placebo for induction of remission. However, patients on thiopurines could reduce the consumption of CSs\textsuperscript{46}. Thiopurines are associated with malignancies and even if the total risk is low, a fourfold risk of lymphomas (notably in males) and in non-melanoma skin cancers in individuals <50 years of age has been shown, and thiopurines augments the risk for the much-feared...

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Infliximab (IFX, Remicade®) is a chimeric monoclonal IgG₁ antibody against TNF-α, an important pro-inflammatory cytokine that takes part in intestinal inflammation. Another TNF-α inhibitor is Adalimumab, a human antibody monoclonal drug used against IBD. These drugs are effective in inducing remission in both CD and UC. Moreover, they are sometimes used as a remission therapy, but mostly as a maintenance treatment for patients with severe IBD, especially effective early (<3 month after diagnosis) in the disease course. As recommended by the ESPGHAN/ECCO guidelines, anti-TNF treatment is indicated for patients with luminal disease despite optimal immuno-modulators, for children with active steroid-refractory disease and for Crohn’s patients with active perianal fistulizing disease. One study showed that IFX maintenance therapy was effective, being associated with prolonged CS withdrawal over a 3-year period in children with CD. A new report, evaluating the occurrence of suboptimal IFX therapy in adult patients with IBD, showed that a majority of the patients (64% of UC and 58% of CD) had at least one indicator of suboptimal therapy (dose escalation, discontinuation, switching, non-biologic therapy escalation or surgery). Anti-TNF treatment has big advantages, but also some difficulties, except from the expense. Serious infections; sepsis, meningitis, abscesses, pneumonia, and herpes zoster, were seen in 33% of the patients in pediatric studies in which infections had occurred. Other malignancies or Hemophagocytic Lymphohistiocytosis (HLH) are not associated with anti-TNF treatment in children according to a new study from 2017.

Because IFX is biologically active, the drug is sometimes difficult to control. To maintain satisfactory levels of IFX in the circulation, serum trough levels should be followed (trough level refers to the concentration of a drug in the circulation just before the next administration). If the s-IFX trough level is low, the anti-TNF dosage and/or IFX infusion intervals should be adjusted. Loss of response can occur because of antibodies against IFX and because the inflammatory burden is very extensive. ATI can also produce acute infusion reactions and delayed-type hypersensitivity reactions. Most studies on IFX trough levels of IFX in the circulation, serum trough levels should be followed (trough level refers to the concentration of a drug in the circulation just before the next administration). If the s-IFX trough level is low, the anti-TNF dosage and/or IFX infusion intervals should be adjusted. Loss of response can occur because of antibodies against IFX and because the inflammatory burden is very extensive. ATI can also produce acute infusion reactions and delayed-type hypersensitivity reactions. Most studies on IFX trough levels of IFX in the circulation, serum trough levels should be followed (trough level refers to the concentration of a drug in the circulation just before the next administration). If the s-IFX trough level is low, the anti-TNF dosage and/or IFX infusion intervals should be adjusted. Loss of response can occur because of antibodies against IFX and because the inflammatory burden is very extensive. ATI can also produce acute infusion reactions and delayed-type hypersensitivity reactions. Most studies on IFX trough
The gut microbiota refers to the bacteria, fungi, viruses, protozoa and archaea that inhabit the gut in children. Corticosteroid treatment alone concomitant GMA treatment significantly reduced the amount of inflammation during one GMA session. These immune cells are important producers of large numbers of pro-inflammatory cytokines, including TNF-α, IL-1, IL-6 and IL-8. Accordingly, by reducing the activated leukocytes, the number of cytokines is reduced. Additionally, it reduces L-selectin and the chemokine receptor CXCR3, which mediates migration of leukocytes from blood into the inflamed intestinal tissue.

The GMA method uses intravenous access at two sites. The patient’s venous blood passes through the column and returns to the patient via the second venipuncture in the opposite arm. The flow rate is 30 ml/min and the treatment session lasts approximately 60 minutes. In children, it is traditionally used as last resort treatment. Reports show that the effect on remission is best in steroid-naïve patients and early in the course of the disease. Some studies suggest that more intense treatment (two to three sessions a week instead of conventional once a week) is more effective in inducing remission.

A Japanese study of 40 adult UC patients compared GMA treatment in combination with CSs vs. CS treatment alone. The patients were followed for 5 years. The study showed that concomitant GMA treatment significantly reduced the amount of CSs needed compared with corticosteroid treatment alone. Only a few previous reports are published considered GMA in children.

1.7.6 Granulocyte monocyte apheresis

Granulocyte and monocyte apheresis (GMA) is a device for selective depletion of the innate immune cells and can be used as treatment for both UC and CD. It contains acetaldehyde beads that attract and remove the Fc-γ receptors of activated leukocytes (granulocytes and monocytes) with a granulocyte adsorption ability over 2.18 x 10⁹ cells during one GMA session. These immune cells are important producers of large numbers of pro-inflammatory cytokines, including TNF-α, IL-1, IL-6 and IL-8. Accordingly, by reducing the activated leukocytes, the number of cytokines is reduced. Additionally, it reduces L-selectin and the chemokine receptor CXCR3, which mediates migration of leukocytes from blood into the inflamed intestinal tissue.

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1.8 MICROFLORA

Gut microbiota refers to the bacteria, fungi, viruses, protozoa and archaea that inhabit the gut. The gut is the most heavily colonized organ, harboring over 70% of the microbes in the human body. Over 50 phyla have been described to inhibit the gut but the most predominant phyla of bacteria are Bacteroides and Firmicutes, which are considered strict anaerobes.
The commensals (the beneficial bacterial population in the gut, also termed the normal microbial flora) aid in nutrient metabolism, prevent colonization of pathogenic microorganisms and help maintain healthy intestinal barrier function. The immune system seems to have evolved in coexistence with the commensals and together they defend against invasive pathological microbes. Intestinal colonization starts immediately at birth when the young infant is exposed to various bacteria in the birth canal. Babies delivered by caesarian section exhibit a different gut flora than vaginally delivered babies. It is presumed that the initial colonization shapes the composition of the gut microbiota, which is relatively stable after 1 year of age and resembles that of a young adult.

There are large inter-individual differences in gut flora. The mother’s gut flora composition is known to have the greatest impact on the child’s developing gut flora, but some obesity studies have shown that food may alter the composition of gut microbiota.

An imbalance in the composition of the intestinal bacteria, i.e. symbiosis, is most likely involved in the pathogenesis of IBD. In genome-wide association studies (GWAS), epigenetic findings and other genetic analyses have revealed links between changes in the intestinal microbiota and an improper immune response in IBD patients. This improper response is probably due to differences in the gut microbiota and altered bacterial function. A Swedish twin study showed that gut microbial composition seems to be determined by genetics and environmental exposure in childhood. The Swedish study also showed that CD is associated with subsequent changes in the intestinal microbiota. With new advanced biotechnology that includes modern sequencing and computer analysis methods, it is possible to investigate the microbial composition and characteristics of the bacteria.

1.9 THE HEALTHY INTESTINAL IMMUNE SYSTEM

An intact immune system protects against uncontrolled and ongoing inflammation when confronted with the high antigen load that we are exposed to, both by food and the microbiota. The immune system is divided into the innate immune system and the adaptive (acquired) immune system, representing different layers of function as well as evolutionary origin. The innate immune system includes monocytes, which develop to macrophages, dendritic cells (DC), neutrophils, eosinophils, basophils and natural killer cells (NKCs), the complement system and the cytokines and the recently discovered innate lymphoid cells. The innate immune response is initiated when pattern recognition receptors (PRRs) and toll-like receptors (TLRs) on the cell surface and NOD-like receptors in the cytoplasm recognize microbial antigens.

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Innate immunity is present at birth and provides an immediate response to foreign invaders. Unlike the adaptive immunity, no specific antigen is remembered and no protection against future infection and invasion is provided.

The adaptive immune system is not present at birth but develops as the immune system meets foreign antigens, adapts and remembers. T and B lymphocytes are responsible for acquired immunity; the immune response begins when antibodies produced by B lymphocytes encounter an antigen and the cytokines and the complement system enhances the ability of the antibodies to clear microbes and damaged cells from an organism.

1.9.1 Intestinal microbiota and oral tolerance

Immune homeostasis refers to the highly complex interactions between inflammation and immunogenic tolerance to maintain balance in the immune system. This process starts in the healthy gut with oral tolerance, which is closely tied to the commensals. In the newborn baby, the gut flora is sterile (in utero), the milieu is free of microorganisms, but colonization with commensals starts during birth, with aerobic species dominating at first, followed by anaerobes. During this early microbial colonization, the mucosal immune system matures. Oral tolerance is a very important maturity function of the intestinal immune system exerted by the commensal bacteria that modulate the gene expression linked to crucial functions: xenobiotic metabolism (metabolism of toxins and drugs), mucosal barrier strengthening, nutrient absorption, intestinal maturation and angiogenesis. The mechanisms for its establishment and maintenance are incompletely understood.

1.9.2 Epithelial barrier

In the intestinal immune system, first-line defense is represented by the epithelial barrier with an intestinal epithelium built up by one layer of cells, with interspersed, mucin-secreting Goblet cells. Figure 2. The mucus layer harbors commensals and is covered with secretory IgA and a glycocalyx (a coat of glycolipids, glycoproteins that contribute to cell-cell recognition, communication and intercellular adhesion). Paneth cells are a specialized type of cells in the small intestine and produce various antimicrobial proteins, comparable with a broad-spectrum antibiotic against both gram positive (gram+) and gram negative (gram-) bacteria. They also seem to have an important role by secreting factors that help in the regulation of the epithelial stem- and progenitor cell who replenish the epithelial cells in the small intestine. The Paneth cells secrete the antimicrobial protein α-defensin (small cysteine-rich cationic proteins). White blood cells also have a role in the immune system, they are the cells found in the blood that fight infection and are responsible for the immune response. They include lymphocytes, neutrophils, and monocytes/macrophages.
blood cells and mucosal epithelial cells in entire GI tract secret β-defensins, another antimicrobial protein. The defensins exert their effect through lyse bacterial membranes with their amphipathic properties.\textsuperscript{123,124}

1.9.3 Antigen recognition and immune regulation

The luminal epithelium expresses PRRs who recognize several microbial components or microbe-associated molecular patterns: lipopolysaccharides, peptidoglycans, lipoteichoic acid and single stranded and double stranded RNA and methylated DNA that are unique to microbes. One important PRR group in mammals is the TLRs that are capable of recognizing most molecular patterns of the microbes.\textsuperscript{120} TLRs trigger both innate and adaptive responses to microbes initiating an intracellular signaling process that results in a cytokine cascade leading to inflammation.\textsuperscript{125} When antigen-presenting cells (APCs) encounter peptidoglycans (exposed on the surface in some microbes), cytosolic NOD1 and NOD2 proteins are expressed, whereby pro-inflammatory cytokines are released and further contribute to the innate immune response.\textsuperscript{126,127} Conversely, TLRs have been shown to contribute to intestinal homeostasis when not triggered by pathogens.\textsuperscript{128,129}

1.9.4 Dendritic and lymphoid cells

Many types of immune cell are found in the mucosa, including T cells, B cells, granulocytes and NKC. The microfold cells (M cells) in the villus function as channels where antigens (including microbes) can reach the Peyer’s patches (small intestinal lymph nodes) and the lymphoid follicles (colonic lymph nodes), where they meet APCs such as dendritic cells and macrophages.\textsuperscript{130} DCs express all types of TLR and NOD, enabling them to distinguish between commensals and pathogens and to either activate or silence T cell responses, which balances the immune response.\textsuperscript{131} In healthy persons, T cell unresponsiveness is induced by the DCs that sample antigen and thus display an immature phenotype that stimulates naïve T cells to differentiation into regulatory CD4+ T cells rather than effector Th1 or Th2 cells. When DCs sense pathogens, they mature, become activated and induce immunity.\textsuperscript{132} This process represents an important aspect of how the immune homeostasis of the healthy GI tracts is achieved.
1.9.5 Cytokines

Cytokines refer to a broad category of small signaling proteins, approximately 5-20 kDa, that participate in the immune responses as immune-modulating agents, and are produced by macrophages, monocytes, T-cells, B-cells, DCs, NK-cells, bone marrow stromal cells, epithelial cells and fibroblasts. They take part in cell activation, growth, and differentiation, thereby the cytokines play an important role in the inflammatory process and immunity. The first cytokine was identified 1957. Interferons (IFN), interleukins (IL), chemokines, mesenchymal growth factors (MGF), the tumor necrosis factor family (TNF) and adipokines are described. Cytokines exert their function thorough autocrine signaling, paracrine signaling (from one cell to a nearby cell) and by endocrine signaling (via the circulation to a distant cell) and bind to a specific receptor. Thus, cytokines are able to mediate a variety of functions (pleiotropic) like recruitment of leukocytes and complex intracellular signaling involved in the inflammatory response. The nomenclature is traditionally built from their function, divided into pro-inflammatory, regulatory and anti-inflammatory. Nevertheless, new reports suggest that at least the regulatory cytokines may have variable functions and are able to promote as well as hamper inflammation, depending on the immunological condition and timing. An overview over the complex cytokine landscape in table 5.

1.9.6 Chemokines and chemokine receptors

Chemokines (chemoattractant cytokines) are a subgroup of cytokines (8-12 kDa), produced by different cells in order to recruit leukocytes to the sites of inflammation (chemotaxis). The chemokines are divided into four families, CXC, C-C, C and CX3C, depending on where the N-terminal cysteine residues. Chemokine signals are transduced through binding to members of the seven-transmembrane G protein–coupled receptor (GPCR), and chemotaxis is induced when chemokines binds to the target cell with varying affinity to either one or several of their connected chemokine receptors (CCRs). To date, 23 CCRs that may exert chemotaxis have been identified, together with some 50 chemokine ligands. The CCR system is complex and promiscuous; a single receptor has multiple chemokine ligands whereas a single chemokine binds to several receptors. Besides the chemotactic function, chemokines induce angiogenesis and additionally seem to be involved in T-helper cell differentiation either directly or indirectly by cytokine secretion or by alter the APC trafficking.
1.10 THE INTESTINAL IMMUNE SYSTEM IN IBD

In patients with IBD, several immunological dysfunctions seem to prevail, involving both the innate and adaptive immune system. They appear to have reduced epithelial resistance and increased permeability in the mucosa with a subsequent leaky epithelial barrier12. T-cells mediated disruption of the tight junction and dysfunction of the enteric neuron are the supposed mechanism for this permeability23. Figure 2. Increasing evidence has shown that a disturbed innate immunity takes part in the pathogenesis of both CD and UC. Paneth cell defects in ileal CD leads to depressed expression of α-defensin while the expression of β-defensin is depressed in colonic CD45.

Table 5. Cytokines, the main source, the target cells and the function of the cytokine.

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<th>TARGET CELL(s)</th>
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Mφ=macrophage, Th= T helper cell, NK= natural killer cell, DC= dendritic cell, BMM= bone marrow

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NOD2 is an intracellular PRR encoded by the NOD2 gene (chromosome 16). It recognizes peptidoglycans in the cell wall of bacteria and stimulates immune responses. Reduced NOD2 function may be followed by the inhibition of TLR stimulation, leading to extreme Th1 response through different inflammatory pathways \(^{144}\). Patients with NOD 2 and Atg16L1 mutations also seem to have impaired autophagy function, which is one of the immune dysfunctions seen in IBD. Bactericidal effects and presentations of antigens start the autophagy process in the cell and thus the lack of autophagy leads to excessive amounts of activated immune cells \(^{145}\). This event is due to a failure of central (thymic) and peripheral tolerance \(^{146}\).

The epithelial cells in a healthy person express TLR3 and TLR5 (TLR 2 and TLR4 are difficult to detect). In patients with CD, but not in UC patients, TLR3 is significantly down regulated, whereas in both CD and UC patients TLR4 is strongly upregulated \(^{147}\).

It has also been shown that the function of DCs is faulty in patients with IBD, both in the recognition and processing of the antigen. They incorrectly get triggered by the commensals and induce a Th1 (and possibly Th17) pro-inflammatory immune response normally restricted to pathogens \(^{148}\). Patients with IBD apparently have more activated DCs in the inflamed mucosa and a lack of tolerogenic DCs in the circulation that correlates with the extent of inflammation \(^{149}\). Individuals with IBD have disturbed apoptosis (programmed cell death), a condition leading to the persistence of hyper-reactive T cells. This event is due to a failure of central (thymic) and peripheral tolerance \(^{146}\). Overall, in active IBD the balance between effector T cells and regulatory T cells is disturbed. The effector Th1 and Th2 T cells predominate over regulatory T cells because of naïve T cells (T0), preferably differentiating into Th1 (in CD) \(^{149}\). CD is considered driven by a Th1 immune response, whereas UC is known as a Th2-mediated disease \(^{146}\). The activated effector T cells produce pro-inflammatory cytokines that stimulate macrophages to secrete large amounts of TNF-α, IL-1 and IL-6, thus enhancing the inflammation. The tissue damage is additionally exerted by IL-12-activated NK cells who secret pro-inflammatory cytokines and exercise a direct cytotoxic effect on their target cells. In addition, a large number of leukocytes enter from the intestinal microcirculation, releasing chemokines that attract more activated leukocytes. The vicious circle of both dysfunctional and upregulated innate and adaptive immune responses is thus perpetuated. \(^{120}\)
Figure 2. The epithelial barrier and response to invading pathogens. Ref Metha et al.
2 AIMS AND OBJECTIVES

General aim

The overall aim of this thesis was twofold: (1) to study the clinical effect of Infliximab in children on maintenance treatment, and of granulocyte and monocyte apheresis and exclusive enteral nutrition in children with new onset IBD and (2) to examine the immunological profile in blood at onset and in intestinal mucosa in children at onset and post-treatment.

Specific objectives

- To study trough s-IFX and ATI to identify any correlation with inflammatory activity indices and clinical response in 45 children on maintenance IFX treatment.
- To investigate the clinical effect of GMA and mesalazine as induction of remission in children with new onset IBD colitis.
- To examine the clinical effect of EEN in children with new onset CD.
- To study the mucosal cytokine profile in children with IBD at onset and after induction of remission with GMA and EEN.
- To look into the chemokine receptor pattern on circulating leukocytes in children with CD, UC and healthy controls to determine a prognostic marker in blood to distinguish UC from CD.
### Table 5. Overview of study design, methods and participants

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#### 3.1 STUDY SUBJECTS

All patients in paper I (the IFX study) (n=45, age 7–18 years, CD: n=32, UC: n=13) were enrolled between September 2013 and May 2015. The children were patients at the Gastroenterology Department at Sachs’ Children and Youth Hospital, Stockholm, Sweden, Astrid Lindgren Children’s Hospital, Solna and Huddinge, Sweden and at Västmanland Hospital, Västern, Sweden. Inclusion criteria were patients with UC or CD on maintenance IFX treatment who had received at least three induction doses. Exclusion criteria were any use of other biological treatment.

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The patients in paper II (the GMA study) (n=13) were previously healthy children from 12 to 18 years of age. They were enrolled between December 2012 and April 2016 as a single-center study at Sachs’ Children and Youth Hospital. Inclusion criteria were previously healthy children with newly onset IBD ≥ 12 years of age and a body weight of a minimum of 30 kg. Exclusion criteria was UC with limited inflammatory extension, any use of immunosuppressant drug within six month of the study and a history of anxiety when exposed to intravenous cannulation. They were primarily diagnosed with UC according to the ECCO/ESPGHAN criteria for diagnosis of IBD. However, after histopathological review, in two patients the diagnosis was changed to colonic CD. One patient left the study after five GMA sessions because of non-response. Twelve (92%) patients completed the study according to the study protocol.

In paper III (the chemokine receptor study), 16 UC patients, 12 CD patients and 17 healthy controls were recruited between December 2012 and June 2016. The IBD patients also participated in the GMA study (paper II) and EEN study (paper IV). The patients were diagnosed according to the ECCO/ESPGHAN criteria for diagnosis of IBD and an additional histopathological scoring implemented in the framework of the studies. Additionally, 10 patients’ blood were used in this study from four UC patients who initially were included but left the studies, and in six children six without inflammation immediately after the diagnostic work-up. Eleven children, who were admitted for hand surgery or MRI scan under general anesthesia, served as healthy controls together with the six non-IBD patients described above.

In paper IV (the EEN study), 19 patients were enrolled between August 2013 and September 2016. Of these, 13 were initially diagnosed with CD according to the ECCO/ESPGHAN guidelines. An additional six patients were recruited: one with a juvenile polyp and five without intestinal inflammation, referred to as the non-IBD controls (the same non-IBD controls as in paper III and V). Inclusion criteria were previously healthy children with new CD. Exclusion criteria were any use of immunosuppressant drug within six month of the study. One CD patient left the study after the diagnostic endoscopy. After completion of induction of remission with EEN and histopathological review, two patients had their diagnosis changed to UC.

In paper V, the study population comprised seven patients from the GMA study (paper II, five patients with UC and two with CD colitis) and six non-IBD controls (the same non-IBD controls as in paper IV).
3.2 STUDY DESCRIPTION

3.2.1 Paper I

Forty-five children contributed with one to four blood samples each (in total 93 samples). Serum samples of 2 mL were obtained before scheduled IFX infusion and were analyzed for s-IFX (trough level) and antibodies towards IFX (ATI) using an in-house-developed ELISA. ATI could only be analyzed in samples with undetectable trough levels of IFX (<0.2 µg/mL). At least one sample was obtained in the framework of the study. Additional trough s-IFX already taken at the discretion of the treating physician outside this study was included in the analysis. Dose changes of IFX were not planned in the study. CRP, ESR and albumin were registered at every infusion. FCP was collected in 34/45 (76%) patients. PUCAI and PCDAI were calculated at the time of IFX infusion, except for approximately 20%, which were calculated retrospectively based on charts. CD patients were considered in remission when their PCDAI was <10\(^4\), CRP <5\(^1\) and ESR <10\(^1\); UC patients were considered in remission if the PUCAI was <10\(^4\), CRP <5\(^1\) and ESR <10.

3.2.2 Paper II

Twelve children with newly onset IBD colitis completed 10 GMA sessions with an additional low to moderate dose of mesalazine. Any use of additional treatments was recorded. Three months after complete GMA treatment, a control colonoscopy (CC) was performed. The time interval was chosen because of the suggested time for the development of a steady state leukocyte transmigration between blood and mucosa\(^1\). Blood count, ESR, CRP, albumin, F-calprotectin, endoscopic Mayo scoring, Paris classification, PUCAI and Geboes histological scoring were measured and compared at disease onset and at CC.

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Figure 3. Flow chart of the participants’ way throughout study II, III, IV and V. (The results of the one-year follow-up is not included in the studies presented in this theses)

Diagnostic upper/lower endoscopy with biopsies, analyses of F and blood. Study II, III, IV, V

Histopathological findings, CMCP, chemokine rec. expr., PCDAI/PUCAI, Blood chemistry, F-calprotectin

CD (IV) → UC (II, V)

EEN 6 w → GMA + mesalazine 5 w

Upper/lower endoscopy

Lower endoscopy

Histopathological findings, CMCP, PUCAL, Blood chemistry, F-calprotectin

Upper/lower endoscopy → blood

Histopathological findings, CMCP, PUCAL, Blood chemistry, F-calprotectin

Continued mesalazine or AZA

33
3.2.3 Paper III

This pilot study aimed at finding a prognostic marker in blood to distinguish UC from CD. Peripheral blood samples from children with new onset UC and CD (diagnosed according to the ECCO/ESPGHAN criteria) and from 17 children without IBD (or any other inflammatory condition who served as healthy controls) were analyzed with flow cytometry (LSR Fortessa) that allowed the detection of 20 chemokine receptors on lymphocytes (CD3+), B cells (CD19+), monocytes (CD14+) and granulocytes (CD16+). Expression levels for each CCR on the respective leukocyte population were calculated. A diagnostic algorithm based on these markers distinguished UC from CD in >92% of the studied cases. The disease characteristics were classified, and laboratory values as well as histopathological characteristics were described at onset.

3.2.4 Paper IV

Twelve children who were initially diagnosed with CD were treated with a polymeric EEN for 6 weeks. Short time after completion of EEN, a CC was performed. Weight, height, albumin, blood count, ESR, CRP, F-calprotectin, SES-CD, Paris classification, PCD/AI and Geboes histological scoring (with additional characteristics for CD) were measured at diagnosis and at CC. The colonic mucosal cytokine pattern (CMCP), measured by real time polymerase chain reaction (PCR), was investigated in six CD patients at diagnosis and at CC; in the remaining seven patients, the CMCP was analyzed either at diagnosis or at CC; in the six non-IBD controls, the CMCP was analyzed at the initial colonoscopy. The CMCP was compared in the six EEN-treated patients between diagnosis and at CC. In addition, CMCP comparisons were made between the IBD patients and the non-IBD controls.

3.2.5 Paper V

In seven of the GMA treated patients with IBD colitis that participated in paper II, the CMCP measured by real time PCR (see method below), was evaluated and compared at diagnosis and at CC. Additionally, the CMCP was investigated in six non-IBD controls at onset and compared with the children with IBD. Clinical outcome (measured with ESR, CRP, albumin, F-calprotectin, Mayo sum and PUCAI) was compared at onset and at CC. Patient demography and disease extension (Paris scoring) were described at onset in all patients.

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3.3 GRANULOCYTE AND MONOCYTE APHERESIS WITH ADACOLUMN®

In paper II and V, we used the Adacolumn® Apheresis system comprising of Adacolumn® (ADA) (column filled with cellulose acetate beads as the adsorptive carriers), Adamonitor (blood pump) and the Adacircuit (tubing). The blood is drawn into the column from a vein of one arm via a simple venipuncture, is pumped through the ADA and then returned to the patient via a vein of the contralateral arm. The session was performed during 60 minutes at a flow rate of 30 ml/minute with a single intravenous dose of intravenous heparin (5000 E) to prevent clotting of the venous catheter. The participants were given EMLA® cream (a local anesthetic, lidocaine 2.5% and prilocaine 2.5%) at least 1 hour before venipuncture. Figure 4 schematically depicts how the ADA treatment is performed.

Figure 4. The ADA treatment (from Otsuka® Pharmaceutical homepage)

3.4 POLYMERASE CHAIN REACTION (PCR)

In paper IV and V, we investigated the cytokines CSF-2, IFN-γ, TNF-α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12B, IL-13, IL-22, IL-23a, IL-36y, TGF-β1 and one control gene (ABL CT). The amount of mRNA in the 14 pro-inflammatory mediators was compared with the signal from the ABL gene and the result was presented as the ratio cytokine/ABL. Biopsies for the CMCP were put into RNA and kept at +6 °C for 24 hours and then frozen (-20°C) until analysis. Total RNA
was isolated using the Fibrous tissue kit (Qiagen, Hilden, Germany). The defrosted and minced biopsies were homogenized and 70% ethanol was added. The homogenates were loaded to spin columns, centrifuged and the columns were washed with RW1 buffer and then treated by DNase I and washed again with RW1 buffer. The remainder was handled according to the manufacturer’s protocol. cDNA was obtained by reverse transcription. Quantitative real-time PCR (qPCR) was performed using the 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) for quantification. Probes were obtained from Applied Biosystems (TaqMan® MGB probes, FAM™ dye-labeled) according to manufacturer’s protocol. Fold increases of mRNA transcripts were calculated as follows: ΔΔCt = ΔCt sample − average ΔCt control group and fold difference = 2−ΔΔCt. For more detailed information, see paper IV and V.

### 3.5 FLOW CYTOMETRY (FACS)

**In paper III,** we used fluorescence activated cell sorter scan (FACS) analysis of chemokine receptor expression on four leukocytes T-lymphocytes (CD3+), B cells (CD19+), monocytes (CD14+) and granulocytes (CD16+). The leukocytes were isolated from heparinized whole blood samples by incubation in a hypotonic buffer, followed by a blockade of unspecific Fc-receptor interactions by incubation in phosphate-buffered saline supplemented with 10% human serum. This procedure was followed by surface staining using combinations of fluorochrome-labelled antibodies. Isotype and fluorochrome-matched control antibodies were used to define marker positivity. It is a well-established fact that the accuracy and robustness of the flow cytometry method constitute a limitation with regards to separating negative populations from those that are dimly positive. This can be due to experimental assumptions in the antibody conjugate staining protocols, as well as day-to-day variations in the optical performance of the flow cytometer. Therefore, we excluded those variables that had a measured isotype-control normalized MFI of <300 in both disease groups from the analysis (Table 1) and 52 variables were excluded from further analysis. FACS analyses were performed on an LSR Fortessa cytometer (BD Biosciences). The raw data files from FACS analyses were imported into FlowJo software (Treestar Inc.), which was used for all data generation. For more detailed information, see paper III.
3.6 STATISTICAL ANALYSES

**Paper I:** The correlation between s-IFX (dependent variable) and ESR, CRP, albumin and clinical scoring (independent variables) were assessed with linear regression using the standard error to test univariate associations. Other intergroup comparisons were performed with two-tailed t-tests. A simple linear regression was conducted to correlate changes in dose to changes in trough IFX. In all statistical analyses, one outlier (IFX trough level 40 µg/mL) was excluded.

**Paper II:** The Shapiro Wilks test was used for normality testing in all parameters and comparisons between ESR, CRP, albumin, Hb, calprotectin and Mayo endoscopic score before and after treatment were conducted with paired sample t-tests. The McNemar test was used to compare correlations of PUCAI before and after ADA treatment.

**Paper III:** First, statistical exclusion criteria were established to reduce the number of analytical variables and remove chemokine receptors that did not have predictive value. Logistic regression was then used and 28 univariate logistic regression analyses were performed. These 28 univariate logistic regression analyses revealed four chemokine receptors (CXCR4 (CD10), CXCR1 (CD16), CCR9 (CD14), CCR4 (CD14)) that had a corresponding p-value of <0.1. Those four chemokines were then put into a multiple logistic regression model. The likelihood ratio test revealed that the model had a p-value of p=0.0033. The model managed to separate the data correctly in 92.31% of the cases in the dataset. See paper III for more detailed information of the logistic regression models.

**Paper IV and V:** The Shapiro-Wilks test was performed to test normal distribution in clinical chemistry values, weight, height, SES-CD (paper IV), Mayo endoscopic score (paper V) and clinical scoring. Comparisons before and after treatment were performed for all parameters with paired sample t-tests (if parametric) or Wilcoxon paired t-tests (if non-parametric). Cytokine profiles before and after treatment were performed with the Wilcoxon paired t-tests and Mann-Whitney U test to compare cytokine profiles between groups.

All statistical analyses were performed with IBM SPSS Statistics 23 Data Editor® software (all papers) and Stata 13.1 (paper I). Statistical significance was set at p<0.05.
3.7 ETHICAL APPROVAL
In all studies, patients aged ≥15 years and the parents/caregivers to children < 15 years gave written informed consent to participate in the studies before any study-related procedure was initiated in accordance with the Helsinki II Declaration. All studies were approved by the Regional Ethics Committee in Stockholm, Sweden (No. 2011/1927-31/2, No. 2010/1252-31/1 and No. 2012/378-31/3).

4 RESULTS

4.1 S-IFX TROUGH LEVELS, ATI AND CORRELATION TO INFLAMMATORY MARKERS
In Paper 1, the children had received a mean number of 13 IFX infusions (range 4-48, mean interval 44.8 days (SD ± 11.2, range 3.44-10.5)) and a mean IFX dose of 6.4 mg/kg (SD ± 1.7) mg/kg was measured. The patients showed clinical remission (PCDAI <10 or PUCAI <10) at 44 of 93 visits (47%). When clinical remission was defined with a stricter definition, including clinical scoring <10, CRP <5 and ESR <10, the patients showed remission only at 26 of 93 visits (28%).

The mean s-IFX trough level was 5.2 µg/mL (median 4.5 µg/mL, range 0.2-21), showing a significantly higher s-IFX level during remission (mean 7.2 µg/mL) compared with during active disease (mean 4.5 µg/mL) (Figure 5).
ATI was found in 12 samples from eight children. All of them were in active disease at every sample occasion. Furthermore, in six additional children, s-IFX was detectable but <1.0 µg/mL and all but one of these were in active disease.

When the patients in active disease and remissions were compared, no significant differences were evident in dose interval (43 days for patients in active disease vs. 42.7 days in remission, p=0.88) or in mean IFX dose (6.4 mg/kg for children in active disease vs. 6.5 mg/kg in remission, p=0.76). Additionally, no clear correlation was seen between IFX trough levels and intra-individual variations in dosing (mg/kg/infusion intervals in days).

IFX trough levels and disease activity were associated, showing negative correlations with clinical indices (p=0.0259), CRP (p=0.0084), ESR (p=0.0035) and a positive correlation with albumin (p=0.00059).

4.2 GMA AND MESALAZINE AS INDUCTION OF REMISSION IN CHILDREN WITH NEWLY ONSET IBD
In paper II, 12 of the 13 participants completed 12 GMA sessions over a median period of 6.25 weeks (range 4.5-8) with an additional dose of mesalazine (39-65 mg/kg). One patient
One more child received CS due to a flare of 12 patients, 5 still used mesalazine but had never required any supplemental IBD treatment. Geboes score) was seen in two patients and improvement in and the other two showed no mucosal healing.

Laboratory values: Laboratory values were measured at onset and at CC. Significant decreases were seen in Hb (p=0.002), albumin (p=0.019) and F-calprotectin (p=0.005); a non-significant decrease was observed in ESR (p=0.058), whereas no decrease was noted in CRP (p=0.133).

Mucosal Healing: After the 10<sup>th</sup> ADA session, CC was performed in all children at a median of 93 (range 62-122) days. The mean Mayo score based on four segments was 1.75 at onset and 0.75 at CC (p=0.006). Of the three patients treated with prednisolone, one had mucosal healing and the other two showed no mucosal healing. Complete mucosal healing (according to the Geboes score) was seen in two patients and improvement in an additional five patients.

One-year follow-up: When more than one year had passed (34-48 month) after inclusion for 10 of 12 patients, 5 still used mesalazine but had never required any supplemental IBD treatment. One more child received CS due to a flare several months after CC in addition to the three.
children treated with CS due to flares between GMA and CC. The CS treated children and additionally one patient (not treated with CS) received arathiopine as maintenance treatment.

4.3 CHEMOKINE RECEPTOR EXPRESSION ON BLOOD LEUKOCYTES

**Paper III:** By profiling the chemokine receptors on circulating T-lymphocytes (CD3+), B cells (CD19+), monocytes (CD14+) and granulocytes (CD16+), the expression pattern of CCR9, CCR4, CXCR1 and CXCR4 enabled a clinical distinction between UC and CD in >92% of the patients.

4.4 CLINICAL OUTCOME OF EEN

In **Paper IV**, 12 children, 10 with CD and 2 with UC, completed 6 weeks with an EEN (polymeric diet) without the need of a feeding tube. No adverse effects were noted, except for mild stomach pain in two patients that could be ignored.

**Disease activity:** A significant decrease was found in PCDAI at CC (p=0.02): median PCDAI at inclusion was 26.5 and only 5 after EEN induction treatment. Ten of 12 patients (83%) showed clinical remission (PCDAI <10) after EEN induction treatment.

**Laboratory tests and anthropometric data:** Significant decreases were found in ESR, CRP and F-calprotectin and significant increases in albumin, Hb, height and weight (Table 7).

<table>
<thead>
<tr>
<th>Table 7. Laboratory values, weight and height at inclusion and at control colonoscopy</th>
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<tr>
<td><strong>At inclusion</strong></td>
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<tr>
<td><strong>Value</strong></td>
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<td><strong>Hb (g/dL)</strong></td>
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<td><strong>Pl (mg/dL)</strong></td>
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<td><strong>Albumin (g/L)</strong></td>
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<td><strong>K.C. (kg/m²)</strong></td>
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<tr>
<td><strong>F.crisis (mg/kg)</strong></td>
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**Mucosal healing:** The CC was performed at a median of 13 (range 1-45) days after completed EEN. In comparison with the diagnostic endoscopy, a significant decrease (p=0.008) was noted in SES-CD after complete EEN. The mucosal biopsies were histologically scored using the...
4.5 COLONIC MUCOSAL CYTOKINE PROFILES AND CLINICAL OUTCOME

The colonic mucosal cytokine profiles (CMCP) were measured in biopsies of the EEN-treated patients (paper IV) and in the GMA-treated children (paper V). Due to circumstances beyond our control, the CMCP was not investigated in all the participants who participated in the studies included in this thesis.

In six EEN-treated patients, paper IV, who completed 6 weeks of EEN, decreases as well as increases in the cytokine expressions were seen in individual patients. In most pro-inflammatory cytokines decreases were seen, and in the anti- and regulatory cytokines, both decreases and increases were observed.

We also compared the CMCP in the available biopsies from inclusion (n=8) and in the available biopsies after EEN treatment (n=7). Non-significant decreases were seen in IL-1β (p=0.064) and IL-23α (p=0.064). When the CMCP in six non-IBD controls was compared with that of the eight IBD patients at inclusion, significantly higher values of IL-12β (p=0.007) and IL-23α (p=0.025) were observed in the IBD patients.

In paper V, seven patients treated with GMA and mesalazine indicated significant decreases in CSF-2 (p=0.018), TNF-α (p=0.028), IL-23α (p=0.043), IL-1β (p=0.028), IL-36γ (p=0.018), IL-10 (p=0.028) and TGFβ1 (p=0.043). For IL-4 (p=0.068), IL-5 (p=0.068) and IL-6 (p=0.075), a decreasing trend, though not statistically significant, was observed after induction treatment. No significant differences could be detected in IFN-γ (p=0.176), IL-12β (p=0.499), IL-22 (p=0.398) and IL-13 (p=0.138).

Looking at comparisons between IBD patients and non-IBD controls at disease onset, the CMCP showed significantly higher IL-12β (p=0.023) and IL-23α (p=0.046) in the children with IBD. A higher expression of IL-22 (p=0.088) and IL-36γ (p=0.062) was found in the IBD patients but without reaching statistical significance.

In paper V, clinical outcome was also investigated showing a median PUCAI of 50 (IQR 40-70, range 30-80) at inclusion; at CC, there was a significant improvement in PUCAI (median 0, IQR 0-15, range 0-25) (p=0.001). The endoscopic Mayo scoring showed additionally significant improvement (p=0.013) between inclusion (median 7, IQR 6-9, range 6-12) and CC (median 3, Geboes score with an addition for CD characteristics. The Geboes score showed improvements in 10 patients, worsening in 1 and no inflammation in 1.

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which differs from our study in which the patients reflect an everyday clinical setting.

Another reported 1.9 µg/mL at week 30 and 2.6 µg/mL at week 46 when receiving 5 mg/kg IFX during remission (mean 7.2 µg/mL, and yet another reported 1.9 µg/mL at week 30 and 2.6 µg/mL at week 46 when receiving 5 mg/kg). A comparatively high mean dose of IFX was found (6.4 ±1.7 mg/kg) and a mean s-IFX trough level of 5.2 µg/mL (range < 0.2-21), with a much higher s-IFX during remission (mean 7.2 µg/mL) compared with the children in active disease (mean 4.5 µg/mL) (p<0.05). Two previous reports on s-IFX trough levels in children described a median s-IFX of 3.5 µg/mL, and yet another reported 1.9 µg/mL at week 30 and 2.6 µg/mL at week 46 when receiving 5 mg/kg. The children in these studies were subjected to active therapeutic drug monitoring (TDM) which differs from our study in which the patients reflect an everyday clinical setting. TDM

5 GENERAL DISCUSSION

The general aim of the theses was to investigate the clinical outcome of three CS free treatments and the subsequent immunological effects exerted in blood and colonic mucosa.

IBD in children consists of a broad spectrum of phenotypes, and does not only refer to the two entities (CD and UC), but also how the disease behaves in the individual patient. Given the difficulties in correctly diagnosing patients, the possibilities to optimize the treatment might be limited. Indeed, prognostic markers, a growing arsenal of treatments and tools to even better evaluate treatment outcome are challenges for the future. Another problem in pediatric IBD care is the wide use of CSs. As a pediatrician, it is highly unsatisfactory to give the child a treatment you know most certainly will cause side effects and probably not compel the child into mucosal healing.

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5.1 IFX TROUGH LEVELS AND ANTIBODIES TO IFX

In paper I, our aim was to correlate s-trough levels and ATI to clinical activity and response in 45 children (CD: n=32 and UC: n=13) on maintenance IFX treatment. Our findings revealed surprisingly low numbers of patients in remission. When clinical scoring was measured (PUCAI or PCDAI <10), the children were in clinical remission in 44 of 93 visits (47%). However, when a stricter definition of remission was applied (CRP <5, ESR <10 and clinical scoring <10), clinical remission was observed in only 26 of 93 visits (28%). In line with other reports, we found statistically significant correlations between s-IFX and clinical indices, as well as with CRP, ESR, and albumin levels, with the exception for F-calprotectin. A comparatively high mean dose of IFX was found (6.4 ±1.7 mg/kg) and a mean s-IFX trough level of 5.2 µg/mL (range < 0.2-21), with a much higher s-IFX during remission (mean 7.2 µg/mL) compared with the children in active disease (mean 4.5 µg/mL) (p<0.05). Two previous reports on s-IFX trough levels in children described a median s-IFX of 3.5 µg/mL, and yet another reported 1.9 µg/mL at week 30 and 2.6 µg/mL at week 46 when receiving 5 mg/kg. The children in these studies were subjected to active therapeutic drug monitoring (TDM) which differs from our study in which the patients reflect an everyday clinical setting. TDM...
refers to the clinical practice of measuring specific drugs at designated intervals to maintain a constant blood concentration of the drug in order to optimize individual dosage regimens\(^{56}\).

Loss of response to anti-TNF is common. Several studies have shown that only 25–41% of adult patients have a sustained response to anti-TNF treatment after 1 year\(^{157, 158}\), showing better results in children. De Bie et al. showed that the probability of losing response to IFX in a pediatric population was 13%, 40% and 50% after 1, 3 and 5 years, respectively. In another study, Vahabnezhad et al. found that in children with CD, 88% remained on IFX at 1 year, 80% at 2 years and 82% at 5 years\(^{159, 160}\). In our report, low s-IFX was associated with the formation of ATI, which was found in 12 samples from eight children, all in active disease. Loss of response was first described by Baert et al. and was demonstrated to be closely correlated to ATI\(^{161, 162}\). Most studies, including our study, have used a method which allows ATI to be measured only in the absence of IFX. Nevertheless, Vande Casteele et al. showed (their method allowed for the analysis of ATI in the presence of the drug) that the presence of ATI, even at therapeutic concentrations of IFX, increased the probability of active disease\(^{163}\). It is conceivable that the same condition prevails in the pediatric population. To monitor the patients thoroughly seems to be a very important factor in IFX treatment outcome. The current strategy is to treat the patients with an induction treatment at week 0, 2 and 6, of 5 mg/kg and a subsequent maintenance treatment of approximately 5 mg/kg, and wait with a dose-escalating until the patients prove to have active disease in combination with sub-therapeutic drug concentrations\(^{40, 101, 164}\). This approach probably allows for formation of ATI which may lead to decreasingly concentrations of the drug and finally results in loss of response. Many factors, except of ATI, have been described to influence the clearance of IFX; gender, disease phenotype, body size, albumin concentration and concomitant immunomodulators\(^{165}\). The pivotal SONIC study investigated the associations of IFX, immunomodulators and remission, and found that 96 of 169 (56.8%) adult patients who received IFX and concomitant immunomodulators were in corticosteroid-free clinical remission at week 26 compared with 75 of 169 patients (44.4%) receiving IFX alone\(^{166}\). In children, Grossi et al. repeated these results, and showed that IFX treated CD patients on concomitant immunomodulators for > 6 months after starting IFX, increased the chances to remain on IFX\(^{167}\). Interestingly, in our study, only 28 of 93 samples (30%) were collected at the time of concomitant immunosuppression. Mean trough IFX was 6.5 µg/L (0.2–21) compared with 4.8 µg/L (0.2–14) in samples from patients on monotherapy (n = 65, 70%) without reaching a significant difference between the two groups.

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In order to optimize the management of s-IFX trough levels, investigations of s-IFX during induction treatment have been studied. One pediatric study investigated the s-IFX in 77 patients in week 10 of induction, and yet in another study of 291 adult IBD patients, as early as after 2 weeks of induction. These induction periods proved effective in predicting 1-year outcome (trough levels at week 10) and for short term and medium term clinical efficacy in UC patients (trough levels at week 2) but in CD, week 2 trough levels were only associated with short term clinical outcomes. A new way of optimizing the anti-TNF dosing is described by Dubinsky et al. by using a prototype dashboard to compare forecasted dosing regimens with actual administered regimens and a standard of care regimen (5–10 mg/kg and an interval every 6–8 weeks). Fifty children completed induction treatment and maintenance treatment (weeks 14 – 54) and clinical and laboratory values, S-IFX and ATI were measured at week 14 and 54. They found that the dashboard recommended dose and/or interval changes in 48/50 patients and that standard of care dosing was recommended in 11/50 patients. The authors conclude that dashboards will be an important tool to individualize the IFX treatment for achieving a durable remission.

Other mechanisms of loss of response are also important to recognize. Low albumin values are known to correlate to low s-IFX. Brandse et al. elegantly showed IFX loss through the feces, and even more, that patients with low albumin concentrations had higher fecal IFX concentrations at day 1 and subsequently lower s-IFX at week 2. This finding probably reflects a general protein loss followed by an extensively ulcerated intestinal surface.

A limitation of our study is the small number of participants and the use of a retrospective design. The strength may be that our study reflects the reality of patients not being subject to active TDM. Therefore, conclusions about the role of active TDM-based IFX treatment were beyond the scope of this report. In summary, a very active and alert approach (which was not the case in our study cohort) with early and ongoing s-IFX trough levels for dose optimization, additionally thorough work-up with inflammatory parameters and clinical scoring, as well as an active decision if concomitant immunomodulators is suitable, seems to be a wise strategy to maintain response to IFX treatment.
Why were only one third of the patients in remission? Many patients were tested for s-IFX for the first time when they participated in this study. Are children without TDM without control? Is this very expensive treatment not what we hoped for? Is it ethical to keep the patients on maintenance treatment without remission? And in that case how should the remission be evaluated?

Do doctors have too strong reliance on anti-TNF? If that is the case, is it because of the limited arsenal of treatments, or is it connected with the pharmaceutical industry and how anti-TNF has been presented?

Practical matters: If I had known, I would have talked to the statistician or some other skilled person about how to enter the data in the best way directly into the statistical program. That would have saved me from a lot of extra work.

### 5.2 GMA For Induction of Remission and Changes in Mucosal Cytokine Profiles

In paper II (a pilot study), we flipped the traditional treatment algorithm by using the most often last treatment option: GMA, in combination with a low to moderate dose of mesalazine, as induction of remission treatment to newly onset IBD colitis patients. Twelve children, 10 with UC and 2 with CD colitis, completed 10 GMA sessions according to the study protocol. Our findings revealed an effective treatment with a significant decrease in PUCAI, F-calprotectin and ESR and a significant rise in Hb and albumin. Additionally, nine children achieved endoscopic significant remission (Mayo scoring) and two of these were in complete histologic remission (Geboes scoring). To the best of our knowledge, only one report on GMA as early induction therapy in the treatment of naïve children has been published. Tanaka et al. included 24 children with newly onset UC who received 5-ASA or sulphasalazine treatment. Seventeen of the 24 children were non-responders and were then treated with 11 sessions of GMA followed by clinical evaluation and control endoscopy. GMA was associated with clinical remission and mucosal healing in 12 of the 17 GMA monotherapy-treated patients. In non-responders to GMA monotherapy (five patients), an additional low dose of Prednisolone enhanced the efficacy of the GMA therapy. Furthermore, tapering of the Prednisolone dose soon after remission was not associated with UC relapse\textsuperscript{195}. The authors discuss the importance of giving GMA early in the disease course. Previous studies (20 to 40 patients included) have shown that steroid naïve (adult) patients with short duration of UC are the best responders to GMA treatment, especially compared with patients with deep colonic lesions and extensive loss of the mucosal tissue at UC lesion. Further, that the amount of used CS was significantly lower in CS treated patients who received additional GMA treatment\textsuperscript{196, 199, 178-179}.
In 2003, Tomomasa et al conducted the first pediatric study on 12 steroid refractory children in active UC who were treated with one GMA session for 5 to 10 weeks. Eight of these patients achieved significant clinical and endoscopic remission\(^{173}\). This study is followed by a handful of pediatric reports. Runuska et al, for instance, showed significantly reduced steroid dosage in 37 steroid-dependent children after five to nine GMA sessions\(^{175}\). In addition, some other, small studies (four to nine patients) have reported encouraging results\(^{175, 179}\).

Based on the study protocol, the participating children in our study were diagnosed with UC according to the ECCO/ESPGHAN criteria\(^{44}\). After pathology review of the biopsies, two patients had a change in diagnosis to CD colitis. According to the largest genotype association study to date, in which the biological relations between CD and UC were studied, Cleynen et al. suggest that three distinct groups (ileoal CD, colonic CD and UC) better explain the phenotypes in IBD. The authors further propose that this nomenclature should be used in a clinical context.

Based on this recommendation, we did not exclude the CD patients from the study. In fact, it turned out that the CD patients were good responders to the induction of remission treatment\(^{24}\).

In paper V, we investigated the (CMCPs) in 7 (five with UC and two with CD) of the 12 GMA patients who participated in paper II. The cytokine expression was investigated with real-time PCR at onset and after treatment in the seven GMA patients as well as in six patients without intestinal inflammation (non-IBD controls). Fourteen cytokines (pro-inflammatory, anti-inflammatory and regulatory) were measured. After induction treatment, significant decreases were noted in CSF-2, TNF-α, IL-23a, IL-1β, IL-6, IL-10 and TGFβ1 and non-significant decreases in IL-4, IL-5 and IL-6. We also found significant higher levels of IL-12p2 and IL-23a as well as non-significant higher values of IL-22 and IL-36y between the IBD patients and the non-IBD controls at onset. ESR, F-calprotectin, PUCAI and the endoscopic Mayo score were found to significantly decrease after treatment compared with onset values; a significant rise in serum albumin was also observed. To our knowledge, this is the first report on CMCPs after GMA with mesalazine for induction of remission in pediatric patients. There are only a few reports in this area on adult patients. Yamamoto et al. studied the clinical and endoscopic outcome as well as cytokine profiles after GMA in 28 adult patients\(^{177}\). They found significant decreases in IL-1β, TNF-α and IL-6, which agrees with our results for children. Velikova et al. reported upregulated gene expression of several cytokines in 37 adult patients with active IBD and higher gene expression in the IBD patients compared with 12 non-IBD controls. They also correlated the cytokine gene expression of the IBD patients to different anti IBD treatments and found that Azathioprine ± 5-ASA or CSs were more beneficial than 5-ASA to restore immune
regulation. One limitation of paper II and IV is the small sample size, but a more serious limitation is that the results mirror the mesalazine treatment alone. We used GMA together with mesalazine for medico-ethical reasons to avoid the risk of giving the patient a non-effective treatment. Romano et al. studied the effect of high-dose mesalazine (80 mg/kg) remission treatment in 15 pediatric UC patients of whom 10 were newly diagnosed. Endoscopic remission at week 12 was found in 4 of the 15 children, which may support the notion that mesalazine alone would not have been sufficient as induction of remission in the present study. Indeed, the children in our study all suffered from extensive or pan-colitis and would probably have been treated with CsIs in a standard care setting. Three children also received CsIs between the 10th GMA session and CC because of flares, and two of these were still in active disease at CC despite the CS treatment. The investigation of the CMCPs in children after GMA and mesalazine is an unexplored research area with no available studies for comparison. A known mechanism of GMA is the removal of activated blood leucocytes, cells known to be important producers of pro-inflammatory cytokines. Thus, we speculate that the clinical efficacy mirrors the decreased cytokine expression after GMA and mesalazine as induction of remission, and given the low level of side effects, this is a highly favorable treatment in children with IBD. Nonetheless, we recommend a larger controlled clinical trial comparing GMA and mesalazine against CS as an induction of remission in treatment naïve children with IBD colitis.

The GMA study was exciting to perform. The patients/parents were very content with the CS free treatment, and so was I.

I know that many colleagues do not have confidence in the GMA treatment. And of course, no big RCTs have been performed except from one that was made on adult patients in active disease who were on concomitant treatments, far from treatment naïve children. Is it an effective strategy to eliminate the cytokine producing cells before they reach the site of inflammation? If the answer of that question is yes, GMA is probably an effective treatment.

Besides no big RCTs, I believe that the expense of GMA makes it difficult to implement GMA in the healthcare. Is it ethical to NOT perform a head-to-head RCT on GMA vs CS? After all, this is a well-tolerated non-steroid remission treatment.

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5.3 CHEMOKINE RECEPTORS ON BLOOD LEUKOCYTES AS A PROGNOSTIC MARKER FOR CD AND UC

Paper III is a pilot study that aimed to find a prognostic marker for UC and CD in blood at disease onset. We set out to characterize cell surface chemokine receptor (CCR) expression on the leukocytes in blood from children in the GMA (paper II and V) and EEN study (paper IV) and in non-IBD controls. We identified four CCRs: CCR9, CCR4, CXCR1 and CXCR4, whose expression pattern enabled a clinical distinction between UC and CD in >92% of the cases in our study cohort. Presumably, this pilot study is the first to investigate whether CCRs can be used as a prognostic marker for pediatric UC and CD. There is an urgent need for more accurate and less invasive diagnostic methods. It can be difficult to diagnose children with newly onset IBD, where a mistaken diagnosis could lead to either an incorrect treatment or an inferior one followed by delayed remission. EEN is suggested as the first-line therapy to induce remission in children with CD, whereas in UC the first-line treatment is traditionally CSs44, 45. In a Swedish register-based cohort study the diagnosis for many patients changed during the follow-up period. According to unpublished data (Olén et al), about 3% of the children primarily diagnosed with CD had their diagnosis changed to UC, approximately 10% diagnosed with UC had their diagnosis changed to CD and almost 45% diagnosed as IBD-U had their diagnosis changed to either CD (36%) or UC (19%). The IBSEN study showed that in 9% of the cases the diagnosis changed between the entities79. Even more troubling is that a delayed diagnosis may be followed by a lag in pubertal growth spurt and impaired adult height189. Studies investigating non-invasive diagnostic markers in children with IBD are limited. One study showed that the presence of a specific volatile organic compound (VOC pattern) in the exhaled breath discriminated between IBD patients and controls191. Monasta et al. further explored this issue with a case-control study (pilot) using ion molecule reaction-mass spectrometry and studied whether the alveolar air of 67 children with IBD (33 UC and 34 CD) presents a specific VOC pattern when compared with controls (65 controls with GI complaint and 102 healthy controls)192. The authors found that pediatric IBD patients (and CD patients in particular) have different alveolar air VOC patterns compared with healthy children and gastroenterological controls. They also noted that CD and UC present different VOC patterns.

A limitation of our study is the small sample size, followed by the important question concerning whether the results are applicable to the IBD population in general. The participants in our study were investigated according to the ECCO/ESPGHAN guidelines44, with a

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EEN FOR INDUCTION OF REMISSION AND CHANGES IN MUCOSAL CYTOKINE PROFILES

EEN is the standard treatment for children with CD according to the European guidelines, but the mode of action is not elucidated. In study IV, we aimed to investigate whether the effect of EEN as induction therapy was paralleled by changes in the CMCP. Eleven of 12 (92%) children completed 6 weeks of EEN, apart from one child who completed only 4 weeks. The children received a polymeric nutritional drink (Fortimel Energy®) that was taken orally by all patients. We found significant decreases in PCDAI, CRP, ESR, SES-CD and F-calprotectin and a significant increase in Hb, albumin, weight and height after induction of remission with EEN as a monotherapy. Ten of the 12 patients (83%) achieved clinical remission and histological scoring showed improvement in 10 patients, worsening in one and no inflammation in one.

It is well-known that EEN is an equally effective remission treatment compared with the previously used first-line treatment (i.e. CSs), but EEN is superior in achieving mucosal healing. Furthermore, one study showed nearly equal effectiveness between anti-TNF and EEN in clinical outcomes (PCDAI) and mucosal healing as estimated by fecal calprotectin. Nevertheless, a new American review with the aim to assess the efficacy of EEN was initiated because CSs are still frequently used to induce remission in pediatric CD despite the potential adverse events in children. They found no difference in efficacy between EEN and CSs, and that a greater proportion achieved mucosal healing than the CS treated children, thus the same result as previous reports. It has previously been shown that pediatric gastroenterologists in North America found their concern of patient adherence as the main disadvantage with EEN. Svalos et al. investigated the opinion of the use of EEN and alternative novel, solid food-based diets (SFDs) in 29 children previously treated with 8 weeks of EEN (parental attitudes were also measured). They found that while patients with CD and their families would accept an EEN repeat, the majority preferred a SFD. Unfortunately, we did not follow the patients with any questionnaire forms regarding quality of life. However, our overall impression is a good acceptance of the EEN treatment (except for one child who completed only 4 weeks) and thus our results are in concordance with Svalos et al. Except from subsequent histopathological scoring applied within the framework of the study. Thus, the patients had a clear diagnosis of CD or UC. Furthermore, no active selection of the patients was performed and thus the participating patients mirror those patients in a standard clinical setting. We suggest that it would be valuable to further explore the use of CCRs on blood leukocytes as a diagnostic biomarker for CD and UC in treatment-naive children.
two patients who suffered from mild stomach pain during intake of the nutritional drinks, no adverse or side effects could be detected in our study population. We treated the patients with a polymeric nutritional drink because of its better taste that permits oral intake. No significant differences in efficacy have been reported (with endpoint mucosal healing) between elemental, semi-elemental and polymeric formulations. In our study population, and especially in the case of the teenage patients, the offer of a feeding tube was out of the question. Consequently, none of the children used a feeding tube and 11 successfully completed 6 weeks of EEN. Before start of the induction treatment, the doctor had a lengthy conversation with the children and their parents about treatment options and the advantage EEN therapy in terms of its high quality. A dietician had close contact with the patients and their parents throughout the EEN treatment. We believe that the combination of a convincing attitude of the doctor, a close follow-up by the dietician and a polymeric nutritional drink is the best formula to achieve successful EEN treatment.

A particularly noteworthy finding was the good clinical response in the two UC patients (one also had achieved endoscopic response with lower values of SES-CD and F-calprotectin). It is extremely difficult to find reports on UC and EEN treatment. If the new nomenclature according to Cleynen et al is implemented, speculation could take place as to whether patients with Crohn colitis would have any advantage of EEN treatment.

In this pilot study, we additionally investigated the CMCP at onset and after completion of induction of remission in six patients (five with CD and one with IBD-U). We found no significant change in expression of any of the 14 cytokines. Nevertheless, in individual patients decreases and increases were measured in the regulatory cytokines TGF-β1, CSF-2 and in the anti-inflammatory cytokine IL-10. Moreover, decreases were seen in most of the pro-inflammatory cytokines studied. The expression of the known pro-inflammatory cytokine IL-12β was upregulated in four patients and only one showed a decrease. Still, when we compared the IBD patients with the non-IBD controls, significantly higher values of IL-12p70 and IL-23α were observed in the IBD patients. These cytokines play an important role in the pathogenesis of CD. There are only a few reports on the CMCP after EEN treatment. In a pediatric report of 29 children, of whom 17 were treatment-naive, Fell et al found a significant decrease in mucosal pro-inflammatory cytokines (measured with PCR) that was associated with clinical remission and macroscopic and histological healing. Their results are partly in line with our finding of an increased expression of TGF-β1 after EEN and a higher cytokine expression in the IBD patients compared with the non-IBD controls. Yamamoto et al. investigated the CMCP...
(five cytokines) with ELISA (enzyme-linked immunosorbent assay). Twenty-eight adult CD patients completed 4 weeks of an elemental formula. The authors demonstrated that mucosal concentrations of TNF-α, IL-1β, IL-6, IL-8 and IL1 receptor antagonists normalized after treatment. They also investigated CMCP in healthy controls. Their finding is consistent with our findings and those of Fell et al, where significantly higher levels were noted in IBD patients compared with healthy controls. One study investigated mucosal cytokines in biopsies of adult UC patients (n=20), and CD patients (n=35) in two colonic sites (a non-inflamed site and an inflamed site) and in healthy controls (n=54)\textsuperscript{156}. They used PCR and found several cytokines augmented in inflamed biopsies compared with normal biopsies. They concluded that specimens with UC and CD show different independent cytokine profiles, suggesting that this knowledge is important in the development of personalized therapies. The individual pattern of the cytokine expression in our study may reflect the CD patients’ diversity in immunological phenotype\textsuperscript{28, 190}. To conclude, we found EEN to be a highly effective treatment, and believe that an intense engagement between the patients and their physicians and dieticians will increase the probability that the patients will adhere to the treatment. The mode of action is still not clear and believed to be highly complex with immunological and microbial interactions. Nonetheless, a better understanding of the role of both anti-inflammatory and regulatory cytokines as well as temporal aspects requires further studies.

In the EEN study, we planned to investigate the mucosal cytokine profiles and connect the immunological results to the mucosal microbiom, investigated by whole genome sequencing. Unfortunately, it was a delay in the sequencing, and the results are still not ready. I believe that there is need for bigger studies that explore and connect the mucosal immunology with the mucosal microbiom in order to understand the mode of action of EEN.

**Practical matters:** If I were to do the study again, I would involve a biomedical scientist from the start. How to handle the material in the best way is an exclusive knowledge, and a biomedical scientist is the one who knows. Things became unnecessarily complicated when it comes to these matters.

**About writing articles and how to please supervisors and reviewers:** the learning process of writing is fun, challenging and at times frustrating. The manuscript may return with the supervisor’s different opinions, or with the reviewer’s wish that you have to add so much that you have to overstep the word limit. Even if it sometimes was difficult to deal with comments and different opinions, I still feel that the review is a valuable tool for the final result.
6 ETHICAL REFLECTIONS

All studies were approved by the local Ethics Committee in Stockholm, Sweden. All patients over 15 years were informed and gave written consent, and if the child was younger than 15 years, informed written consent was obtained from the parents. Our studies were performed in accordance with the Helsinki Declaration, where children are considered as a vulnerable group.38

“Medical research with a vulnerable group is only justified if the research is responsive to the health needs or priorities of this group and the research cannot be carried out in a non-vulnerable group. In addition, this group should stand to benefit from the knowledge, practices or interventions that result from the research.”

A distinct ethical reflection is always important in the context of research in general, and in pediatric research in particular, due to the fact that children belong to a vulnerable group. Nevertheless, pediatric research is important because results from adult research may not always be applicable to children. Subsequently, not conducting pediatric research could be considered unethical because this might be followed by an inferior healthcare for children.

A quite complicated ethical application is inevitable early in the research process. Instead of just thinking of it as problematic, it can be used as a help to define the project and balance the possible disadvantage for the patient with the importance of the results. Next question is for whom the research will be beneficial; for the subject, an extended group of patients, the community or the researcher? These questions are of immense importance, and clear answers are needed before the patients are involved.

The children in my projects have been subject to at least one extra endoscopy examination in general anesthesia with additionally blood sampling that we usually not perform in the ordinary clinical setting. Further, we tried GMA as a remission treatment which is a non-validated method for induction of remission. As a researcher, a conversation with the child and the parents concerning the implication of participation in the studies have been important, both for the subject and for myself. I have given very clear information that the outcome of the treatment is uncertain, and that it entails discomfort to go through an extra endoscopy with bowel preparation. In the EEN study the patients received the same treatment as they would have received in the clinical setting. Nevertheless, many children decided to participate. I believe that it is crucial that the child feels secure and understand what the participation in the study means, and that he or she feels free to leave the study without the feeling of making the doctor sad or risking an inferior care in the future. The most common answer to my question why they wanted to participate in the studies, was that they wanted to contribute to the knowledge of IBD for the sake of science.

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All studies were approved by the local Ethics Committee in Stockholm, Sweden. All patients over 15 years were informed and gave written consent, and if the child was younger than 15 years, informed written consent was obtained from the parents. Our studies were performed in accordance with the Helsinki Declaration, where children are considered as a vulnerable group.38

“Medical research with a vulnerable group is only justified if the research is responsive to the health needs or priorities of this group and the research cannot be carried out in a non-vulnerable group. In addition, this group should stand to benefit from the knowledge, practices or interventions that result from the research.”

A distinct ethical reflection is always important in the context of research in general, and in pediatric research in particular, due to the fact that children belong to a vulnerable group. Nevertheless, pediatric research is important because results from adult research may not always be applicable to children. Subsequently, not conducting pediatric research could be considered unethical because this might be followed by an inferior healthcare for children.

A quite complicated ethical application is inevitable early in the research process. Instead of just thinking of it as problematic, it can be used as a help to define the project and balance the possible disadvantage for the patient with the importance of the results. Next question is for whom the research will be beneficial; for the subject, an extended group of patients, the community or the researcher? These questions are of immense importance, and clear answers are needed before the patients are involved.

The children in my projects have been subject to at least one extra endoscopy examination in general anesthesia with additionally blood sampling that we usually not perform in the ordinary clinical setting. Further, we tried GMA as a remission treatment which is a non-validated method for induction of remission. As a researcher, a conversation with the child and the parents concerning the implication of participation in the studies have been important, both for the subject and for myself. I have given very clear information that the outcome of the treatment is uncertain, and that it entails discomfort to go through an extra endoscopy with bowel preparation. In the EEN study the patients received the same treatment as they would have received in the clinical setting. Nevertheless, many children decided to participate. I believe that it is crucial that the child feels secure and understand what the participation in the study means, and that he or she feels free to leave the study without the feeling of making the doctor sad or risking an inferior care in the future. The most common answer to my question why they wanted to participate in the studies, was that they wanted to contribute to the knowledge of IBD for the sake of science.
7 CONCLUSIONS

In the IFX study, we found that only one third of the children on IFX maintenance treatment were in clinical and biochemical remission, and that s-IFX was significantly higher during remission compared with active disease. A strong correlation was found between ATI and active disease.

GMA and mesalazine were found to be a safe and effective treatment as an induction of remission in children with newly onset IBD. It seems plausible to speculate that the decreases seen in mucosal cytokines after treatment may explain the observed clinical efficacy.

EEN was an effective treatment in all our patients, also in the two patients with UC. The patients completed the treatment without a nasogastric tube and without side effects. Additionally, a change in the mucosal cytokine profile after induction of remission with EEN was observed.

By investigating the CCRs, we found a possibly prognostic marker for UC and CD. We suggest that it would be valuable to further explore the use of CCRs on blood leukocytes as a diagnostic tool.

By investigating the cytokine profiles in mucosal biopsies, we have extended the knowledge of immunological phenotypes in children with IBD.

We think that an active approach must be applied in the care of children with IBD to achieve and maintain remission.
8 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Crohn's sjukdom (CD) och ulcerös kolit (UC) utgör tillsammans gruppen kronisk inflammatorisk tarmsjukdom, som ofta benämnas IBD, efter engelskans "inflammatory bowel disease". I Sverige är ca 0,65 % av befolkningen drabbad, och ca 10-20 % av de drabbade insjuknar under barnom. I Sverige är sjukdomen således ganska vanlig, och IBD är generellt sett vanligare i den rika delen av världen än i den fattiga.

Uppkomstmekanismerna till IBD är okända. Trots mycket forskning i ämnet är det fortfarande oklart varför man insjuknar. Sjukdomsförloppet kännetecknas av att man kan föhävda särskilda karaktäristika tillhörande de olika sjukdomarna.

fall lyckas inte patienterna genomgå behandlingen eller ens få chansen utan behandlas istället med mediciner. Detta är den gängse behandlingen för barn. Barnet hade enbart mediciner, och tillämpades istället för aktiv inflammationsbehandling och inte mådde bra trots behandlingen, och att de som utvecklade antikroppar mot IFX hade något läkemedel kvar i blodet. Alla dessa var i aktiv sjukdom.

**I studie II och V** utförde vi två kopplade studier som tidigare inte gjorts; barn med som nyinsjuknat i kolit (tjocktarmsinflammation) fick behandling med granulocyt monocytt apheres (GMA), vilket vanligtvis ges som en sista utväg vid behandlingsvikt när man provat allt annat och inget fungerar. GMA är inget läkemedel utan jämförs bäst med dialys, där blodet renas från aktiverade inflammationsceller som cirkulärar i blodbanan som annars skulle vandra ner till tjocktarmen och skapa inflammation. Tolb barn och ungdomar över 12 år, fick 10 behandlingar under 5 veckor samt en mild tilläggsbehandling med meslazin (studie II). Barnen genomgick en kontroll koloskopie ca tre månader efter den tioende och sista GMA behandlingen. Vid den första koloskopin och kontroll koloskopin togs även vävnadsprover i tjocktarmen för undersökning av 14 olika inflammationsämnen (cytokiner, undersöks med en avancerad teknik som heter PCR) för immunologisk testning (studie V). Vi fick resultat på före-efter GMA på sju barn. Vi fann vid kontrollen att 9 av 12 barn var helt bra eller tydligt bättre i sin tarmlämning, och att 8 av 12 barn tyckte de känt sig helt bra (klinisk remission) samt tydliga nedgångar i inflammationsprover i blodet. Vid undersökning av cytokinerna i tarmlämninghinn på sju barn sågs en tydlig nedgång i sju olika cytokiner och mycket högre nivåer av cytokiner sågs mellan sjuka och sex friska kontroller. Vi spekulera i att nedgångarna i tarmlämninghinn förklarar det goda behandlingsvaret.

**I studie III undersökte vi små ämnen i blodet som heter chemokin-receptorer med hjälp av en avancerad teknik som kallas för flödescytometri. Chemokin-receptorer är en slags mycket små proteiner som fungerar som adresslappar på inflammationsceller, vilket gör att de guidar inflammationscellerna dit de ska (platsen för inflammation). Vi undersökte chemokin-receptor landskapet på barn med UC, CD och friska kontroller. En statistisk algorithm utifrån chemokin-receptorer mönstrer på fyra olika vita blodkroppar urtaget ur världen som kunde särskilja UC från CD. Detta är en pilotstudie för att utveckla en prognostisk markör där vi i ca 10 % av fallen har svårt att korrekt diagnostisera UC från CD, vilket gör att vissa patienter kan få en felaktig eller sänkta behandling än om de haft rätt diagnostik från början.

**I studie IV** behandlade vi 12 barn meddiagnostiserade med CD och behandlade dem med flytande kostbehandling, s.k. exklusiv enteral nährstoffbehandling (EEN) vilket innebär att barnet endast dricker näringsdrycker (Fortimel Energy®) under sex veckor utan annan föda eller mediciner. Detta är den gängse behandlingen för barn med CD i Europa och USA, men i många fall lyckas inte patienterna genomgå behandlingen eller ens få chansen utan behandlas istället med höga doser kortison. Tidigare studier har visat att behandlingen är livbrukningsförsiktig och läcker tarmen bättre än kortison. Vi ville undersöka hur våra patienter svarade på behandlingen, samt

allergisk reaktion när man får sin IFX-dos. Vanligtvis får patienterna IFX var fjärde till åttonde vecka som infusion på mottagningen. Vi ville studera hur mycket IFX IBF-patienterna har kvar i blodet när de kom för att få sin nästa infusion (dalvärde). Vi kontrollerade sänkande och CRP, och de fick fylla i formulär hur de mådde (klinisk scoring) Vi fann att en del av patienterna hade aktiv inflammation och inte mådde bra trots behandlingen, och att de som utvecklade antikroppar mot IFX inte hade något läkemedel kvar i blodet. Alla dessa var i aktiv sjukdom.

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hur cytokinerna såg ut i tarmen innan före och efter behandling. Elva barn genomförde sex veckors behandling, ett barn genomförde fyra veckor. Alla barn hade mycket god effekt av behandlingen och tydliga nedgångar sågs i inflammationsprover i blodet, kliniskt status och läkning i tarmen. Cytokinprofilerna visade inga tydliga (signifikanta) nedgångar i hela gruppen med vid analys på de enskilda patienterna sågs nedgångar i de flesta cytokinerna men även en del uppgångar i de cytokiner som reglerar eller hämmar inflammationen. Vi bedömer att ett stort engagemang från doktor och dietist med tät uppföljning samt att använda den mest välsmakande näringsdrycken så barnen slipper sond, är nyckeln till en framgångsrik EEN behandling. Vi tror att EEN ger effekter i inflammationsmönstret i tarmen, men fler studier som undersöker detta behöver göras för att bättre förstå varför EEN är en effektiv behandling.
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Ola Winqvist co-supervisor, and Ludvig Linton at ITH-lab, for FACS analysis and help in understanding immunology.

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Jenny Sedelius (former Ranefors) and Åsa Sädeh Zetterlund, thank you for you amazing work in helping the children to maintain to EEN treatment. Without encouragement and engagement this treatment is difficult. I am so sad that you are leaving us.

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Thank you, my Sachs family! Lena, Karin, Josefine, Jocke, Mattias, Mårta, Susanne, Stina, Eva, Erik, Natalia, Annika, Sauna x 2, Fredrik x 2, Olle (still in the family), Martina, Björn, Helenor, Cecilia, Caroline x 2, Per, Marie, Rebecka, Anders, Josefine, Ulrika and many more! Thank you for the lunch conversations, good times, difficult times, parties, skiing weekends, dinners, weddings…

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Johannes, Jakob, Karolina and Kristina, my wonderful, loving children. You mean everything to me! I love you to the moon and back.

Charlie, thank you for standing by my side and giving me all types of support (not at least it-support) during the work with my thesis. Thank you for being such a wonderful dad to our four darlings. I love that you and me are us!

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therapy in children with disease. Gastroenterology 2014;146:383


Serum-Infliximab Trough Levels in 45 Children with Inflammatory Bowel Disease on Maintenance Treatment

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Abstract: The role of trough serum infliximab (s-IFX) and antibodies toward IFX (ATI) during maintenance treatment remains unclear in children. The aim of the present study was to investigate trough s-IFX and ATI to identify any correlation with inflammatory activity and clinical response in a pediatric inflammatory bowel disease (IBD) cohort. We investigated the s-IFX trough levels in pediatric IBD patients (n = 45) on maintenance IFX treatment. Ninety-three blood samples were collected and demographics, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and albumin were recorded. The mean s-IFX trough level was 5.2 µg/mL. The mean trough s-IFX level was significantly higher in the samples taken during remission (7.2 µg/mL) compared to active disease (4.5 µg/mL, p < 0.05). The trough s-IFX levels correlated with ESR, CRP, and albumin. S-IFX was undetectable in eight of the patients, all with positive ATI and active disease. Surprisingly, clinical and biochemical remission was observed at only 26 of the 93 visits. The correlation between dose variations and changes in trough s-IFX was not evident. In line with studies in adults, the s-IFX trough levels correlated with response to infliximab.

Keywords: inflammatory bowel disease; Crohn’s disease; ulcerative colitis; trough levels; antibodies toward infliximab

1. Introduction

Infliximab (IFX, Remicade®) is a chimeric monoclonal IgG1 antibody against tumor necrosis factor (TNF), a central cytokine in inflammatory bowel disease (IBD). This drug is effective in inducing and maintaining remission in Crohn’s disease (CD) and ulcerative colitis (UC), the two principal entities of IBD [1–3]. Baert et al. identified the potential immunogenicity of IFX in IBD patients, suggesting that patients may develop antibodies toward IFX (ATI), especially with episodic administration of the drug [4].

1.1. INTRODUCTION

Infliximab (IFX, Remicade®) is a chimeric monoclonal IgG1 antibody against tumor necrosis factor (TNF), a central cytokine in inflammatory bowel disease (IBD). This drug is effective in inducing and maintaining remission in Crohn’s disease (CD) and ulcerative colitis (UC), the two principal entities of IBD [1–3]. Baert et al. identified the potential immunogenicity of IFX in IBD patients, suggesting that patients may develop antibodies toward IFX (ATI), especially with episodic administration of the drug [4].
However, 6%–17% of the patients develop ATI even with scheduled treatment [5,6]. The association of ATI and infusion reactions is clear, and several studies demonstrate significantly lower trough serum IFX (s-IFX just before the next infusion) in patients with ATI, explained by increased elimination of IFX [4,7]. Low s-IFX and ATI formation have been associated with loss of response (LOR), but it has been difficult to establish any significant correlation between ATI and clinical parameters other than LOR as shown in a meta-analysis including 10 studies and 668 patients [4,9–10]. Treatment response seems to be more related to drug levels rather than ATI, and a clear correlation has been found in several reports between trough s-IFX and clinical remission, C-reactive protein (CRP), and endoscopic improvement in adult patients [8,31–33]. The role of monitoring s-IFX and ATI, therapeutic drug monitoring (TDM), in the clinic is unclear, and the level of evidence is low given a limited number of studies with small cohorts, the use of retrospective designs, and different methodological approaches [10,14,15]. During induction of IFX, low trough levels have been suggested to support dose escalation in case of poor response [56–58]. In patients with loss of response, TDM may support dose escalation or switch to another drug [7,18,19]. A majority of the TDM studies have been performed in adult patients, and even though there are findings suggesting different pharmacodynamics and kinetics in children, very few studies have been undertaken to assess trough levels and ATI in pediatric cohorts [20–23]. The current study includes 45 children with CD or UC on maintenance IFX treatment. The study aims at correlating clinical activity and response to treatment with s-IFX levels and ATI in children.

2. Results

Forty-five children receiving IFX maintenance treatment were included and patients contributed with one sample for each visit with a total of 93 specimens; 15 patients contributed with one sample, 19 patients with two samples, four patients with three samples, and seven patients with four samples. The median age of this pediatric cohort was 16.0 years (range 7–18). CD was diagnosed in 32 patients (71%) and 13 patients (29%) had UC. In the anti-TNF-treated CD cohort, 27 patients (60%) had colonic or ileocolonic inflammation and 15 children (47%) also had involvement of upper gastrointestinal tract (mucosal inflammation found proximal to the ligamentum Treitz) at diagnosis. A proportion of 84% showed an inflammatory phenotype without stricturing or penetrating disease (B1). Only four patients (13%) had penetrating phenotype (B3) and seven children (22%) presented with perianal disease (P).

Among the UC patients, 11/13 (85%) had extensive colitis or pancolitis (Table 1). A majority of the TDM studies have been performed in adult patients, and even though there are findings suggesting different pharmacodynamics and kinetics in children, very few studies have been undertaken to assess trough levels and ATI in pediatric cohorts [20–23]. The current study includes 45 children with CD or UC on maintenance IFX treatment. The study aims at correlating clinical activity and response to treatment with s-IFX levels and ATI in children.

Table 1. Background characteristics of the patient cohort. IBD, inflammatory bowel disease.

<table>
<thead>
<tr>
<th>Patient Characteristics and Disease Classification</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>45</td>
</tr>
<tr>
<td>Male sex, number (%)</td>
<td>29 (64%)</td>
</tr>
<tr>
<td>Age at inclusion, median (range), years</td>
<td>16 (7–18)</td>
</tr>
<tr>
<td>Age at IBD diagnosis, median (range), years</td>
<td>12 (12–17)</td>
</tr>
<tr>
<td>IBD onset (age)</td>
<td>11–15 years</td>
</tr>
<tr>
<td>0–10 years</td>
<td>51%</td>
</tr>
<tr>
<td>11–16 years</td>
<td>36%</td>
</tr>
<tr>
<td>Immunosuppression (Azathioprine) at any time during the study</td>
<td>29 (64%)</td>
</tr>
</tbody>
</table>

Crohn’s disease e ≥ 32 (13%), Paris classification at diagnosis

| L1 (distal 1/3 of ileum + caecum)                   | 5 (16%) |
| L2 (colon)                                         | 12 (37%) |
| L3 (ileocolonic)                                   | 15 (47%) |
| L4e (upper disease proximal Treitz)                 | 15 (47%) |
| L4d (upper disease distal Treitz)                   | 2 (6%)  |
| B1 (non-stricturing/non-penetrating)                | 27 (84%) |
| B2 (stricturing)                                   | 1 (3%)  |
| B3 (penetrating)                                   | 4 (13%) |
| B2R3 (stricturing/penetrating)                      | 0       |

Ulcerative colitis e ≥ 32 (13%), Paris classification at diagnosis

| L1 (distal 1/3 of ileum + caecum)                   | 5 (16%) |
| L2 (colon)                                         | 12 (37%) |
| L3 (ileocolonic)                                   | 15 (47%) |
| L4e (upper disease proximal Treitz)                 | 15 (47%) |
| L4d (upper disease distal Treitz)                   | 2 (6%)  |
| B1 (non-stricturing/non-penetrating)                | 27 (84%) |
| B2 (stricturing)                                   | 1 (3%)  |
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| B2R3 (stricturing/penetrating)                      | 0       |
The duration of the IFX treatment ranged from 3 to 60 months, and the children had received a mean number of 13 IFX infusions (range 4–48). The mean dose of IFX ± standard deviation (SD) was 6.4 ± 1.7 mg/kg (median 6.2 mg/kg, range 3.44–10.5) with a mean interval of 44.8 ± 11.2 days. The mean s-IFX trough level was 5.2 µg/mL (median 4.5 µg/mL; range from <0.2 to 21), showing a right-shifted Gaussian distribution, as seen in Figure 1. One CD patient in remission with s-IFX 40 µg/mL was excluded from the analysis.

The assessment of disease activity was based on the validated scoring indices Pediatric CD Activity Index (PCDAI) and Pediatric UC Activity Index (PUCAI). The children were in clinical remission at 44 out of 93 visits (47%). With a stricter definition of remission using a combination of low clinical scoring and normalized C-Reactive Protein (CRP, mg/L) and Erytrocyte Sedimentation Rate (ESR, mm/h), the patients were in remission at 26 of the 95 test occasions (28%). Nine children were in remission at all visits, while 28 children were not in remission at any visit (10 of these non-remitters had only one visit). The clinical indices and biochemistry are summarized in Table 2.
remission at all visits, while 28 children were not in remission at any visit (10 of these non-remitters had only one visit). The clinical indices and biochemistry are summarized in Table 2. As shown in Figure 2, s-IFX was significantly higher in samples taken during remission (mean 7.2 µg/mL) as compared with sera collected during active disease (mean 4.5 µg/mL, p < 0.05). No significant difference was observed in dose-interval (days) between patients in active disease and those in remission (mean 43.0 days in active disease vs. mean 42.7 days in remission, p = 0.88) or in mean dose of IFX between the children in active disease (6.4 mg/kg) and those in remission (6.5 mg/kg, p = 0.76).

Table 2. Disease activity parameters at time of sampling. PCDAI, Pediatric Crohn’s disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index; C-reactive protein; ESR, erythrocyte sedimentation rate.

<table>
<thead>
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<tr>
<td>PUCAI</td>
<td>&lt;10 (remission) 10/93 (11%)</td>
</tr>
<tr>
<td></td>
<td>≥10          85/93 (91%)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>Median (range) 2.0 (1–63)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
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</tr>
<tr>
<td>Albumin (g/L)</td>
<td>Median (range) 38 (27–44)</td>
</tr>
<tr>
<td>F-Calprotectin (mg/kg)</td>
<td>Median (range) 884 (15–9066)</td>
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Figure 2. The mean trough s-IFX level was significantly higher in the samples taken during remission (7.2 µg/mL) as compared with s-IFX in active disease (4.5 µg/mL, p < 0.05). Clinical remission was assessed from activity scoring: PCDAI < 10 or PUCAI < 10, ESR < 10, and CRP < 5. One outlier of 40 g/L was excluded.

The trough levels indicated a statistically significant correlation with clinical indices, as well as with CRP, ESR, and albumin levels, as illustrated in Figure 3a–d. No correlation was detected between trough levels and Fecal Calprotectin (FCP, mg/kg), and no difference was noted in s-IFX with CRP, ESR, and albumin levels, as illustrated in Figure 3a–d. No significant difference was observed in dose-interval (days) between patients in active disease and those in remission (mean 43.0 days in active disease vs. mean 42.7 days in remission, p = 0.88) or in mean dose of IFX between the children in active disease (6.4 mg/kg) and those in remission (6.5 mg/kg, p = 0.76).

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trough levels and Fecal Calprotectin (FCP, mg/kg), and no difference was noted in s-IFX trough levels between CD and UC (not shown).

Interestingly, intra-individual variations in dosing (mg/kg/infusion interval in days) did not show any clear correlation with changes in trough levels (µg/mL), as seen in Figure 4. Dose changes were not planned within the framework of the study, and were prescribed by the treating physician at his or her own discretion and not necessarily based on trough levels of s-IFX. The dose variations were also due to changes in weight. In the 30 patients who supplied two to four tests (total 48 samples) we identified 18/48 (38%) tests with decreased dose (mean −22%, SD ±13%), and in 11 of these 18 samples s-IFX decreased. Of these 18 samples, seven represented children in remission. At 22/48 (46%) sampling occasions, there was a dose increase (mean +44%, SD ±52%), and 15/22 tests showed subsequent increased s-IFX. Of these 22 samples, 17 tests represented patients with active disease.

In 12 samples from eight children (seven with CD and one with UC) collected at different sampling occasions, s-IFX trough levels were below detection and all of these samples were positive for s-IFX. Of the eight children, seven were in remission at the time of the ATI positive samples. In six additional patients s-IFX was detectable, but below 1.0 µg/mL, giving a total of 14 patients with s-IFX of <1.0 µg/mL. All but one of these 14 patients showed active disease (CRP ≥ 10 or PUCAI ≥ 10, and/or s-ESR ≥ 40 or FCR ≥ 5 or PUCALI ≥ 10).

Of 14 patients with s-IFX of <1.0 µg/mL, only two children had concomitant immunosuppressives during maintenance treatment. In the whole set of 93 s-IFX trough samples, 28 (30%) were collected at the time of concomitant immunosuppression. Mean trough IFX in these samples was 8.5 µg/L (0.2–21).

**Figure 3.** (a–d) Serum-IFX is correlated with the disease activity of the patients. S-IFX showed a negative correlation with: (a) CRP levels (r = 0.0984, r² = 0.0096), (b) s-ESR (r = 0.0103, r² = 0.0011) and (c) activity scoring PUCAI and PCDAI (r = 0.029, r² = 0.0087), and a positive correlation with: (d) s-albumin (r = 0.0065, r² = 0.0001). One outlier of 40 µg/mL was excluded.
This finding should be interpreted with some caution in the light of the restrictive definition of patients comprising IFX-treated children in the counties of Stockholm and Västmanland. S-IFX trough index as well as normalization of CRP/ESR, only at 26 of the 93 visits during maintenance treatment. Based on combined clinical index activity and biomarkers presented a significantly higher mean median trough of 2.9 µg/mL. Hämäläinen et al. and Hoekman et al. both reported a median s-IFX of 1.9 µg/mL in a mixed pediatric UC and CD cohort, whereas Adedokun et al. reported a median s-IFX of 1.9 µg/mL at week 30 and 2.6 µg/mL at week 46 in a UC population receiving 5 mg/kg IFX q8w [22,24,25]. In the latter study, even a double dose of 10 mg/kg only gave a trough level of 2.9 µg/mL [24].

Surprisingly, the children were in clinical remission, defined as a reduction in the clinical activity index of at least 50% in children [20,21,27–29]. The finding of only 28% remission rate based on the combined bioclinical indices/biomarkers [22,24,25]. In adults, the correlation between serum IFX levels and clinical activity defined as a reduction in the clinical activity index of at least 50% in was not significant [18]. Hämäläinen et al. and Hoekman et al. both reported a median s-IFX of 3.5 µg/mL in a mixed pediatric UC and CD cohort, whereas Adedokun et al. reported a median s-IFX of 3.5 µg/mL in a mixed pediatric UC and CD cohort, whereas Adedokun et al. reported a median s-IFX of 3.5 µg/mL at week 30 and 2.6 µg/mL at week 46 in a UC population receiving 5 mg/kg IFX q8w [22,24,25]. In the latter study, even a double dose of 10 mg/kg only gave a trough level of 2.9 µg/mL [24].

In our study, the children who responded to IFX and who were in clinical remission based on combined clinical index activity and biomarkers presented a significantly higher mean trough level (mean 5.2 µg/mL, median 4.5 µg/mL), even though within the proposed therapeutic interval 3–7 µg/mL for adults [18]. In our study, we found a relatively high trough level (mean 5.2 µg/mL, median 4.5 µg/mL), even though within the proposed therapeutic interval 3–7 µg/mL for adults [18]. Hämäläinen et al. and Hoekman et al. both reported a median s-IFX of 3.5 µg/mL in a mixed pediatric UC and CD cohort, whereas Adedokun et al. reported a median s-IFX of 3.5 µg/mL at week 30 and 2.6 µg/mL at week 46 in a UC population receiving 5 mg/kg IFX q8w [22,24,25]. In the latter study, even a double dose of 10 mg/kg only gave a trough level of 2.9 µg/mL [24].

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Figure 4. Change in dose (%) of IFX (mg/kg/dosing in days) did not show significant correlation with change in IFX trough levels in the 30 children who supplied two to four samples each (p = 0.58). The changes in s-IFX were based on samples obtained between approximately one to thirteen months apart.

3. Discussion

The present study investigated s-IFX trough levels and ATI in a cohort of 45 pediatric IBD patients comprising IFX-treated children in the counties of Stockholm and Västmanland. S-IFX trough concentrations showed a significant correlation with clinical response and inflammatory activity. The intra-individual variations in the trough levels between visits were evident, and there was no clear correlation with dose changes, as seen in Figure 4. Low s-IFX trough levels were associated with the formation of ATI.

To our knowledge, there are only a few reports on IFX trough levels in children. In our study, we found a relatively high trough level (mean 5.2 µg/mL, median 4.5 µg/mL), even though within the proposed therapeutic interval 3–7 µg/mL for adults [18]. Hämäläinen et al. and Hoekman et al. both reported a median s-IFX of 3.5 µg/mL in a mixed pediatric UC and CD cohort, whereas Adedokun et al. reported a median s-IFX of 1.9 µg/mL at week 30 and 2.6 µg/mL at week 46 in a UC population receiving 5 mg/kg IFX q8w [22,24,25]. In the latter study, even a double dose of 10 mg/kg only gave a trough level of 2.9 µg/mL [24].

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remission in this study, including clinical indices as well as normalization of biomarkers. Previous reports on IFX and clinical response in children have been divergent, and some studies suggest high rates of LOR up to 50% in children [20,21,27–29]. The finding of only 28% remission rate based on the 93 recorded visits was surprising, especially in light of the relatively high mean trough level of 5.2 µg/mL in this report. A sub-study of the pivotal Crohn trial with IFX (the ACCENT I trial) revealed median week 14 trough levels of 4.0 µg/mL in patients with sustained response to IFX 5 mg/kg and 1.9 µg/mL in patients without sustained response [30].

We could not detect any clear impact of dose changes on the ≥IFX levels. In this study, the ≥IFX is a mixture of tests taken in the clinic by the treating physician, as well as tests obtained within the current study. Therefore, not every decision to change dosing has been based on ≥IFX levels. Moreover, since the dosing also depends on weight and infusion interval, the variation may not be a result of active dosing decisions.

ATI was found in all patients with undetectable ≥IFX, and all these patients had active disease. This observation may suggest that ATI plays an important role in children with low trough levels and incomplete response to the treatment. Yet, a major limitation in the enzyme-linked immunosorbent assay (ELISA) analysis of ATI is the inability to detect antibodies in the presence of IFX residue, which makes it difficult to speculate about the role of ATI in children exhibiting low but detectable trough levels. Vande-Castele et al. investigated the relationship between IFX concentrations, ATI, and disease activity in 1487 IFX trough serum samples from 485 adult CD patients [26]. Their method allowed for the analysis of ATI in the presence of IFX, showing that ATI even at low as well as therapeutic concentrations of IFX increased the probability of active disease. It is conceivable that the same condition prevails in the pediatric population. The combination therapy with immunosuppressives is thought to reduce the frequency of ATI. We found a numerical trend toward a higher ≥IFX concentration in children on azathioprine, but without reaching statistical significance, probably due to the small number [4,10]. Children were on concomitant immunosuppressant only at 30% of the visits during maintenance treatment. This could probably reflect the fear for hepatotoxic T-cell lymphoma in young male patients [31].

Whether TDM is a valuable tool to maintain remission in patients with IBD is not clear. In a randomized controlled study including 263 adult IBD patients dosing was optimized for IFX trough levels 3–7 µg/mL at the start and the patients were then randomly assigned to either continued TDM-based or clinically based dosing [32]. The study showed no superiority of concentration-based dosing after one year with regard to remission rates. However, TDM-based dosing was associated with fewer flares during the course of treatment. Furthermore, a retrospective study examining the use of proactive TDM in 48 adult patients compared to 79 patients with standard care demonstrated a higher probability of remaining on IFX in patients with ≥IFX trough levels >5 µg/mL [33]. In a study by Minar et al., TDM was evaluated in IFX-treated children with CD who experienced LOR. The authors found that ESRs at the previous infusion were significantly associated with IFX concentrations [23]. The current report is of observational and retrospective nature. Interventions were not implemented within the study and therefore conclusions with regard to the role of TDM-based IFX dosing are beyond the scope of this report. Nevertheless, in this cohort with children not subjected to active TDM, only one third was in clinical and biochemical remission.

4. Materials and Methods

4.1. Patients

Between September 2013 and May 2015 all identified pediatric IBD patients (n = 45, age 7–18 years) on IFX maintenance treatment in the counties of Stockholm and Västmanland were enrolled in this retrospective cohort-study. The inclusion criterion was current maintenance IFX treatment after at least three induction doses for the indication of either CD or UC. Serum samples of 2 mL were obtained before scheduled IFX infusions and analyzed for ≥IFX (trough level) using an in-house-developed assay (ELISA) analysis of ATI is the inability to detect antibodies in the presence of IFX residue, which makes it difficult to speculate about the role of ATI in children exhibiting low but detectable trough levels. Vande-Castele et al. investigated the relationship between IFX concentrations, ATI, and disease activity in 1487 IFX trough serum samples from 485 adult CD patients [26]. Their method allowed for the analysis of ATI in the presence of IFX, showing that ATI even at low as well as therapeutic concentrations of IFX increased the probability of active disease. It is conceivable that the same condition prevails in the pediatric population. The combination therapy with immunosuppressives is thought to reduce the frequency of ATI. We found a numerical trend toward a higher ≥IFX concentration in children on azathioprine, but without reaching statistical significance, probably due to the small number [4,10]. Children were on concomitant immunosuppressant only at 30% of the visits during maintenance treatment. This could probably reflect the fear for hepatotoxic T-cell lymphoma in young male patients [31].

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ELISA. In the study, at least one sample was obtained from each identified child on maintenance IFX and moreover, additional trough s-IFX already taken at the discretion of the treating physician outside this study was also included in the analysis. Therefore, the cumulative number of IFX trough samples for each patient differed (result section). Any dose adjustment was done at the discretion of the treating physician. The clinical information collected from the medical charts included age at IBD diagnosis, type of IBD diagnosis, Paris classification, age at inclusion in the study, duration of IFX treatment, current dose correlated to weight, infusion interval in days, and total number of IFX infusions. Concomitant treatment of immune modulators (azathioprine) was registered. Comorbidity with primary sclerosing cholangitis (PSC), diabetes mellitus, vasculitis, celiac disease, and arthritides were also documented (see Table 1 for patient characteristics).

The sera were separated and handled according to standard operative procedures until analysis. Quantification of serum levels of IFX was performed at the Department of Clinical Immunology, Karolinska University Hospital, Stockholm with ELISA assay used in clinical routine in the Stockholm and Västmanland counties. ATIs were analyzed in samples with undetectable (<0.2 µg/mL) trough levels of IFX. CRP (mg/L), ESR (mm) and albumin (g/L) were registered at every infusion. FCP (mg/kg) was collected in 34/45 patients. PUCAI and PCDAI were calculated at the time of IFX infusion, except for approximately 20% which were calculated retrospectively based on charts. CD patients were considered in remission when the PCDAI was <10, CRP < 5, and ESR < 10 [34,35] . These CRP and ESR samples were missing, and in the case of missing values the assessment of remission or active disease was based on available biomarkers together with the clinical activity index. Dose changes were calculated by dividing dose (mg) with weight (kg) and infusion interval (days).

4.2. IFX ELISA

The trough level concentration in serum samples was measured with an in-house developed and validated ELISA methodology used in clinical routine, which has been described previously [31]. Briefly, the microtiter plates (Nunc Maxisorp F 96, Thermo-Fisher Scientific, Roskilde, Denmark) were coated with 100 ng/mL, 50 µg/mL, and 25 µg/mL of recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) in 0.05 M sodium carbonate buffer pH 9.6. The plates were put on a shaker at room temperature (RT) for 2 h and incubated overnight at +4°C. The plates were washed three times in phosphate buffered saline (PBS) plus 0.05% Tween 20 (blocking buffer) for 1 h at RT. After an additional wash, standard dilutions (0.4-100 ng/mL) of IFX (Schering Plough, Kenilworth, NJ, USA) were added to the plate together with defined IFX-spiked sera (internal controls) and patient samples, diluted 1:500 in blocking buffer, all in duplicates. The plates were incubated on a shaker at RT for 1 h and washed four times followed by the addition of alkaline phosphatase (ALP)-conjugated goat anti-human IgG (Fc-specific) (Sigma) diluted 1:10,000 in a blocking buffer. After incubation for an additional hour on a shaker, the plates were washed four times. Substrate (p-nitrophenyl-phosphate, 5 mg/mL in 1 M diethanolamine with 0.5 mM Mg, pH 9.8) was added and color development was monitored at 405 nm. The concentration of samples and controls was calculated from the standard curve. Lower and upper limits of quantification were 0.2 µg/mL and 50 µg/mL, respectively (compensated for serum dilution 1/500).

4.3. Inhibition ELISA for ATI Detection

ATI were analyzed with an in-house developed ELISA based on the inhibition of binding of labeled IFX to TNF-α coated to the ELISA plate, as previously described [31]. ALP was coupled to IFX using the Lightning-Link kit (Innova Biosciences, Cambridge, UK). The plates were coated as described above and washed three times in PBS plus 0.05% pH, Tween20 and incubated with blocking buffer for 1 h at RT. A standard consisting of goat anti-human IgG (see above) at a final concentration of 1 µg/mL and diluted serum samples were incubated with ALP-conjugated IFX for 1 h at RT. After
an additional wash of the TNF-coated plate, aliquots of standard solutions and samples in duplicates were transferred to the plate, which were then incubated on a shaker for 1 h at RT. After additional washes, substrate (see above) was added and color development at 405 nm was monitored. The results were transformed to percentage inhibition by normalization of the samples’ OD to that of the standard (100% inhibition) using the formula (OD blank – OD sample)/(OD blank – OD standard) × 100. The lower limit of detection was set to the value plus two standard deviations obtained from measurements of normal control sera. ATI could only be detected in the absence of the drug because of interference of IFX in the assay. Thus, ATI detection was limited to patients with undetectable s-IFX (<0.2 µg/mL).

4.4. Statistical Analysis

Correlations between clinical parameters, routine chemistry, and s-IFX were assessed with linear regression with cluster-robust standard error to test univariate associations between clinical variables and s-IFX (dependent). Other intergroup comparisons were performed with two-tailed t-test. The correlation between changes in dose and concomitant changes in trough levels was assessed with simple linear regression (Fahrenheit). Statistical analysis was performed with IBM SPSS Statistics 23 Data Editor® software, and Stata 13.1. Significance was set at \( p < 0.05 \). In all statistical analyses one outlier (IFX trough level 40 µg/mL) was excluded.

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5. Conclusions

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Abbreviations

ALP  Alkaline phosphatase
ATI  Antibodies toward infliximab
CD  Crohn’s disease
CRP  C-reactive protein
ESR  Erythrocyte sedimentation rate
LOR  Loss of response
PBS  Phosphate buffered saline
PCDAI  Pediatric CD activity index
PSC  Primary sclerosing cholangitis
PTCAI  Pediatric UC activity index

References


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ABSTRACT

The aim of the study was to analyze the effect of granulocyte and monocyte apheresis (GMA) with mesalazine for induction of remission in pediatric inflammatory bowel disease (IBD) colitis. The mode of action is by adsorption of excess neutrophils and tumor necrosis factor–producing monocytes and release of anti-inflammatory factors. Granulocyte and monocyte apheresis is a nondrug intervention with an excellent safety profile. Granulocyte and monocyte apheresis is a promising treatment option in pediatric inflammatory bowel disease and deserves further investigation. Controlled trials are warranted to confirm the efficacy of this treatment model.

What Is Known

- Granulocyte and monocyte apheresis is a promising treatment option in pediatric inflammatory bowel disease
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What Is New

- Remission treatment outcome using 10 granulocyte and monocyte apheresis sessions in combination with mesalazine in 12 previously untreated children with first onset inflammatory bowel disease colitis: prolonged effect in 8 of 12 patients

Granulocyte and Monocyte Apheresis for Induction of Remission in Children With New-Onset Inflammatory Bowel Colitis

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Granulocyte and monocyte apheresis (GMA) in children with newly onset chronic inflammatory bowel disease (IBD) colitis was effective in 8 of 12 patients. A final diagnosis, however, indicated ulcerative colitis in 10 children and Crohn disease in 2 children. At median colonoscopy, 8 of 12 diarrhea was in clinical remission and the Mayo endoscopic score showed significant improvement in 8 of 12 patients (P < 0.001). Complete mucosal remission according to ileal/facula score was observed in 3 patients. There is only 1 previous report on granulocyte and monocyte apheresis for induction of remission in pediatric inflammatory bowel disease.

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fecal calprotectin (F-calprotectin), and stool cultures for sedimentation rate (ESR), iron status, C-reactive protein (CRP), and investigations: blood count, liver enzymes, albumin, erythrocyte sedimentation rate (ESR), mm 25 (17.5–42) 7–58 9 (3–20) 2–74 0.058

Endoscopy
In line with the ECCO/ESPGHAN guidelines for newly onset IBD in children, all patients were investigated under general anesthesia with upper endoscopy and colonoscopy with multiple biopsies from the esophagus, corpus, antrum, and from the proximal and distal descending colon, ileum, cecum, ascending, transverse, descending, and sigmoid colon and rectum (48). We aimed at a control colonoscopy with multiple biopsies under general anesthesia 12 weeks after the 16th (and final) OMA treatment. This time interval was chosen because of the suggested time for the development of a microbiome-like translocation between bowel and mucosa (22). The baseline Pediatric Ulcerative Colitis Activity Index (PUCAI), disease extension (Paris classification) and endoscopic scoring (Mayo) were registered (9,30) (Supplemental Digital Content 1, Table 5, http://links.lww.com/MPG/B812). The mucosal biopsies were reviewed by an experienced pathologist using the Geboes scoring system (11), which is a commonly used histological score in UC. For simplicity, we express the proportions (percentage) of the patients' biopsies scored: <3 (no IBD inflammation) and ≥3 (IBD inflammation of varying degrees).

Pediatric Ulcerative Colitis Activity Index
The PUCAI encompasses abdominal pain, rectal bleeding, stool consistency, number of stools per 24 hours, nocturnal stools, activity level during the past 2 days. A PUCAI <30 is interpreted as remission, 31 to 54 as mild disease, ≥55 to 84 as moderate disease, and ≥85 as severe disease (16). Significant improvement is considered as a change in PUCAI of ≥35; modest improvement ≥28 to 34, and small improvement ≥20 to 29 (12).

Medical Management
All children received remission treatment with mesalazine (the main active metabolite of 5-ASA) in 12 patients. Before this study, 2 patients were treated with sulfasalazine, 3 children with salazopyrine, 3 children with mesalazine and 5-ASA. We continued on sulfasalazine, 3 children with salazopyrine, 3 children with mesalazine and 5-ASA. We continued on sulfasalazine, 3 children with salazopyrine, 3 children with mesalazine and 5-ASA. The mucosal biopsies were reviewed by an experienced pathologist using the Geboes scoring system (11), which is a commonly used histological score in UC. For simplicity, we express the proportions (percentage) of the patients' biopsies scored: <3 (no IBD inflammation) and ≥3 (IBD inflammation of varying degrees).

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Patient Demography

Half (11 of 22) of the children and adolescents, with verified UC by endoscopy and histopathological examination, entered this study. At diagnosis, all patients experienced extraintestinal colitis (Paris E3–E4). One of 13 had mild disease (PCAI 10–16); 10 of 13 had moderate disease (PCAI 35–44); and 2 of 13 had severe disease (PCAI ≥ 85). Patients was found in 13 to 15 patients and extraintestinal colitis in 2 to 15 (Table 1). One patient with pancreatitis (PCAI score 60) withdrew after 5 ADA sessions due to nonresponse. There were no complications found in the 15 patients, except for anemia in 1 patient.

Adacolumn Treatment and Adverse Effects

The study protocol was designed for 2 ADA sessions per week during 5 weeks. We, however, had to adjust admittments determined by social factors. Four patients received ADA twice a week during 5 weeks and patients twice a week except for 1 week, in which case they were given 5 treatments. Two patients received ADA twice a week for 1 treatment treatment 5 weeks. One patient received HP adhesions over an 8-week period and experienced ADA adhesions once and twice a week until completion of the 10 sessions. The median time needed to complete 10 ADA sessions was 6.25 weeks (range 3.9–10). Twelve patients underwent all 10 ADA sessions and were shown a worsening of symptoms during the treatment period. Three patients had a fall after 4, 11, and 62 weeks of the treatment period. One patient had a fall after 6 weeks, and another patient after 62 weeks. The median time needed for a complete course of 10 ADA treatments was 6.25 weeks. One patient experienced severe worsening of symptoms after 5 ADA sessions and left the study when admitted to the hospital. One patient withdrew from the study after 5 sessions due to nonresponse. There were no adverse effects related to the ADA treatment.

Disease Activity

Pediatric Ulcerative Colitis Activity Index

All patients were classified according to the PCAI and regarded as having active disease at inclusion (Table 2). The rationale for using the PCAI for the 2 patients that had a change of diagnosis to Crohn’s colitis was that the Pediatric Ulcerative Colitis Activity Index did not reflect the clinical symptoms of UC. One patient had mild disease (PCAI 10–16), moderate disease (PCAI 35–44), and severe disease (PCAI ≥ 85). Patients was found in 13 to 15 patients and extraintestinal colitis in 2 to 15 (Table 1). One patient with pancreatitis (PCAI score 60) withdrew after 5 ADA sessions due to nonresponse. There were no complications found in the 15 patients, except for anemia in 1 patient.

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Mucosal Healing

All children were subjected to full endoscopic evaluation of the colon at inclusion and control colonoscopy (median 25 days, range 26–32; after the last ADA session). The endoscopic Mayo score was described in the right segment, in transverse colon, in the left segment and in rectum. The mean Mayo in the right segment was 1.5, in transverse colon 2.1, in the left segment 2.2, and in the rectum 1.5. A mean Mayo score was also calculated for the entire colon based on these 4 segments (21). This showed mean Mayo score 1.75 at the first colonoscopy and 0.87 at the control colonoscopy (P < 0.006). The change in Mayo score between diagnosis and control colonoscopy was shown in Table 3.

One-year follow-up

When >1-year (34–40 months) had passed after inclusion for 10 of 12 patients, we still used the initially prescribed immunosuppressants but never required any supplementary IBD medication. Cile included. One additional patient needed methotrexate at 11 months post-GMA. In addition to the 3 children, who were treated with PSL, due to a flare between ADA and the control endoscopy, 1 more child received PSL, due to a flare several months after the control colonoscopy. These 4 patients were then treated with methotrexate as maintenance treatment.

TABLE 3. Paris score, Mayo endoscopic score, and Geboes histological score in 12 patients at diagnosis and at control colonoscopy

<table>
<thead>
<tr>
<th>Paris Score</th>
<th>Mayo Score</th>
<th>Geboes Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td>Control</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>Paris E3</td>
<td>Mayo 1.5</td>
<td>Geboes 10</td>
</tr>
<tr>
<td>E4</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>E5</td>
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<td>6</td>
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<td>E6</td>
<td>7</td>
<td>6</td>
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<tr>
<td>E6*</td>
<td>7</td>
<td>6</td>
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<td>E7</td>
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<td>5</td>
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<td>E8</td>
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<td>E8*</td>
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<tr>
<td>E9</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>E10*</td>
<td>6</td>
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(21) – Credited disease; SD – standard deviation; E4 – elevated colon.

*Predominant treatment between the 50-50 AG and the endoscopy.

4
DISCUSSION
We investigated 3 therapeutic naive pediatric patients with newly onset IBD extensive or total pancolitis treated in the efficacy of GMA, as a remission induction treatment. Twelve children completed the study, of whom 10 were eventually diagnosed with UC and 2 with CD.

The results demonstrate a significant decrease in PCUAI and 5-ASA consumption and a decrease in both macroscopic and microscopic inflammation. No patient experienced flares during the ADA treatment period. Thirty of 12 patients were, however, given PSL for 1 flare each during the period between the last ADA treatment and the control colonoscopy. Nine of 12 patients achieved endoscopic significant remission (Mayo scoring), and 2 of those patients were in complete histological remission.

There is only 1 similar study on GMA as early induction therapy for children with newly onset UC. Tsuji et al investigated 24 therapeutic naïve children who were given either mesalazine or sulfasalazine for 4 weeks. Nonresponders received 5-ASA monotherapy, and those who did not respond to 5-ASA monotherapy received GMA in combination with CsA. The timing of the control endoscopy in the study varied. Seventeen patients failed remission treatment with mesalazine or sulfasalazine, 12 achieved remission with 5-ASA monotherapy, and 5 achieved remission with GMA in combination with CsA.

Other pediatric trials have examined GMA in children with IBD, who were steroid-dependent or in active disease. Martin de Corpur et al (24) studied 6 children with UC and 4 with CD. After GMA sessions, of 4 patients with UC and 3 with CD, clinical remission was achieved, which was maintained for 1 year in 2 of 4 patients treated with UC and 1 of 3 with CD. Ramsdell et al (25) studied 8 children with BD showing steroid-dependent or active disease. They were treated with 6 to 9 GMA sessions. The authors also thank Carina Hellberg, Sofia Spjuth, and Nadia Asberg for expertise technical assistance and santiabadi AR, Hanai H, Takeuchi K, et al. Adacolumn, an adsorptive carrier based granulocyte and monocyte apheresis device for the treat... Carrier based granulocyte and monocyte apheresis device for the treatment of inflammatory and refractory diseases associated with leuko... 2014;7:48–59. 5. Saniabadi AR, Hanai H, Takeuchi K, et al. Adacolumn, an adsorptive carrier based granulocyte and monocyte apheresis device for the treatment of inflammatory and refractory diseases associated with leuko... 2014;7:48–59.

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REFERENCES
Chemokine receptors on blood leukocytes: a potential diagnostic tool in children with inflammatory bowel disease

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Author contributions

Study concept and design: YF, OW, ME; acquisition of data: LL, HR, MH; data analysis and interpretation: LL, HR, MH; drafting of the manuscript: LL, HR, MH, YF, ME; obtained funding: OW, YF, ME; statistical analysis: MH; clinical study management: HR, YF

Funding sources

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Conflicts of Interest

OW is the founder of, and LL and MH employees at, Immune Therapy Holdings AB, which has co-funded the study. ME has received consultancy honoraria from Immune Therapy Holdings AB. The other authors declare no conflicts of interest.

Abstract

In IBD, reliable diagnostic separation between ulcerative colitis (UC) and Crohn’s disease (CD) is clinically important. Diagnostic misclassification may prevent optimal treatment and there is a need for more accurate and less invasive diagnostic methods. Through a profiling approach of chemokine receptors on blood leukocytes, we have identified four candidate receptors, CCR9, CCR4, CXCR1 and CXCR4, whose expression pattern may be used to clinically separate UC from CD. By defining a diagnostic algorithm based on these markers, we could distinguish UC from CD in >92% of the studied cases in a cohort of 28 treatment-naïve children with newly diagnosed IBD.
Introduction

The clinical distinction between the two main entities of inflammatory bowel disease, ulcerative colitis (UC) and Crohn’s disease (CD), is important for optimizing treatment regime and surgical procedures. Approximately 10-25% of IBD patients are diagnosed before 21 years of age and in several aspects, childhood-onset IBD may be regarded as more complex compared to adults; the clinical course is often more aggressive and the disease more difficult to treat compared to adults.\textsuperscript{1,2} Pediatric IBD may also bring developmental consequences such as puberty delay and growth retardation.\textsuperscript{3}

An accurate and timely diagnosis of UC or CD is important for the individual patient. In making the distinction, the combination of clinical, endoscopic and histopathological evaluation is currently gold standard. In patients with colon-restricted inflammation that lack Crohn’s-associated hallmarks such as transmural inflammation and granulomas, separating CD from UC may be particularly difficult and there is a high risk of misclassification in these patients.\textsuperscript{4} However, in one study as many as 21% of the children receive a diagnosis of unclassified IBD and the IBSEN study demonstrated a change in diagnosis in 9 % of the patients classified as UC and CD.\textsuperscript{5} Thus, there is a need for improved diagnostic precision in general, and non-invasive serological approaches in particular.

Chemokine receptors (CCRs) belong to the G protein-coupled (GPCR) 7-TM superfamily and play an important role in cellular migration during leukocyte development, circulation and tissue relocalization during active inflammation. Through binding to their cognate chemokine ligand, surface-bound CCRs induce cellular migration through an intrinsic signaling pathway which eventually results in actin and myosin filament polymerization. This may change the shape of the cell, allowing it to move in the direction of the chemokine gradient.\textsuperscript{6}

To date, some 20 CCRs have been identified together with approximately 50 chemokine ligands.\textsuperscript{7}
The nature of an underlying immune response has been shown to be reflected by the chemokine receptor expression patterns of migrating leukocytes. Our group has previously demonstrated that a subset of pro-inflammatory blood monocytes is shifted during active adult IBD, potentially reflecting relocation to the gut. These findings support the existing notion of the important role of the receptor-ligand interactions within the CCR system in fine-tuning and balancing immune responses.

In this study, we hypothesized that the CCR expression profile on blood leukocytes would reflect inflammatory stage and phenotype in pediatric IBD patients, and therefore hold diagnostic potential. Thus, we set out to characterize surface CCR expression on blood leukocytes from children with either ulcerative colitis (n=16) or Crohn’s disease (n=12), as well as healthy pediatric controls (n=17) (patient demography is outlined in Supplemental Table 1). All children were investigated and diagnosed according to the ESPGHAN/ECCO guidelines for pediatric IBD (diagnostic characterization is further outlined in Supplemental Table 2). A flow cytometric method was developed, which allowed for the detection of 20 chemokine receptors on lymphocytes (CD3+), B-cells (CD19+), monocytes (CD14+) and granulocytes (CD16+), yielding 80 variables in total. Flow cytometry panels are outlined in Supplemental Table 3. Expression levels for each chemokine receptor on the respective leukocyte populations were calculated by subtracting the median channel fluorescence (MFI) obtained for each isotype-matched control antibody from its corresponding CCR antibody conjugate (Supplemental Figure 1). The method was subsequently applied on heparinized whole blood samples from the pediatric IBD patients (n=28) as well as age-matched healthy controls (n=17), following leukocyte preparation through red blood cell lysis. The samples were analyzed using an LSR Fortessa flow cytometer (BD Biosciences). A statistical method was subsequently developed to reduce the number of chemokine receptor variables which allowed us to identify the most predictive algorithm for UC versus CD. It is a
well-established fact that the accuracy and robustness of the flow cytometry method constitute a limitation with regards to separating negative populations from those that are dimly positive. This can be due to experimental assumptions in the antibody conjugate staining protocols, as well as day-to-day variations in the optical performance of the flow cytometer. Therefore, we excluded those variables that had a measured isotype-control normalized MFI of <300 in both disease groups from the analysis (Table 1) and 52 variables were excluded from further analysis.

Due to the large number of variables in relation to the relatively limited sample size, the material was not suitable for multivariate analysis. Thus, we performed univariate logistic regression analyses on the remaining 28 variables to assess their predictive potential. This approach was chosen due to the non-normality of our data set as well as the binary outcome. The univariate logistic regression analyses ultimately revealed four variables with p<0.1 between the disease groups. Those were CCR9 and CCR4 on CD14+ monocytes; CXCR1 on granulocytes; and CXCR4 on CD3+ T lymphocytes. A multiple logistic regression model was subsequently applied to illustrate what a theoretical predictive model could look like (Figure 1C), resulting in a p-value of p=0.0033 based on the likelihood ratio test. The model managed to separate the data correctly in 92.31% of the cases in the dataset.

In this study, we have addressed the feasibility of using chemokine receptor expression of blood leukocytes as a predictive biomarker for inflammatory bowel disease in treatment-naïve children. We suggest that this accessible diagnostic alternative should be cross-validated in a larger cohort of pediatric IBD patients.
Figure legends

Table 1. Descriptive data summary of the chemokine receptor variables included in this study. Green text represents variables that were included in the proposed predictive model; red text represents variables that were excluded due to the MFI >300 criterion (Supplemental Document 1). Due to the high amount of outliers, Median Absolute Deviation (MAD) was used to present the spread of the data, calculated by following formula: $MAD = \text{median} (x_i - \text{median}(x))$.

Figure 1. Biomarker formulation from chemokine receptor expression profiling of blood leukocytes in juvenile IBD. (A) Spider web charts showing group medians of normalized MFI values for the investigated chemokine receptors in juvenile patients with ulcerative colitis (top row; n=16) and Crohn’s disease (bottom row; n=12). Columns represent the respective blood leukocyte subsets as indicated. Axis labels and MFI scale are outlined in the legend (top right corner). Flow cytometry gating strategy is outlined in Supplemental Figure 1. (B) Scatter plots showing the individual normalized MFI values for the four chemokine receptors with a predictive value of $p<0.1$ between the UC (n=16) and CD (n=12) groups, as defined by univariate logistic regression. Age-matched healthy controls (n=17) are included as references. Bars represent group median values. (C) Equation representing the multiple logistic regression model used to model disease probability.


Supplemental Figure 1. Flow cytometry gating strategy. The flow cytometry gating strategy used to define peripheral blood-derived CD16-positive granulocytes, CD3- and CD16-positive lymphocytes, and CD14-positive monocytes, respectively. In each blood sample, normalized median fluorescence intensity (MFI) values were generated for each studied chemokine receptor in all four individual lymphocyte populations. These were obtained by subtracting

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Supplemental Table 1. Patient demography. Demographics, disease extent and disease activity index at inclusion.

Supplemental Table 2. Diagnostic characterization. Diagnostic characteristics (results of ileocolonoscopy, histopathological examination, blood characteristics and fecal chemistry).

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References

Figure 1

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\[ \log(-1.871 + 0.003 \cdot \text{CXCR4}^{CD3} - 0.00021 \cdot \text{CXCR4}^{CD16} + 0.000889 \cdot \text{CCR5}^{CD14} + 0.0033 \cdot \text{CCR4}^{CD14}) \]
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Materials and methods

Patients

28 children with early-onset inflammatory bowel disease (UC=16; CD=12) were included in the study, together with 17 non-IBD controls. Included patients were children (≤18 years of age) who fulfilled the ESPGHAN/ECCO criteria of ulcerative colitis and Crohn’s disease.1,2 Patients who had received any immunosuppressant medication within the last six months prior to inclusion were excluded from the study.

The non-IBD control group (n=17) was comprised of 6 children in whom investigation for suspected IBD excluded intestinal inflammation; as well as 11 children who were admitted for day care hand surgery of MRI scan under general anesthesia. The control patients had no signs of IBD or other autoimmune disease.

Patient demography is outlined in Supplemental Table 1.

Diagnostic examinations

All patients were investigated according to the ESPGHAN/ECCO guidelines for pediatric IBD with initial laboratory tests; blood count, albumin, erythrocyte sedimentation rate (ESR), C-Reactive Protein (CRP), fecal calprotectin, and stool cultures to exclude infectious diarrhea.

The children underwent upper diagnostic endoscopy and an ileo-colonoscopy with multiple biopsies under general anesthesia.

All patients were characterized according to the Paris classification of disease extension (outlined in Supplemental Table 2).
Flow cytometry

Leukocyte isolation

In order to prepare for flow cytometric (FACS) analysis of chemokine receptor expression, leukocytes were isolated from heparinized whole blood samples by incubation in hypotonic buffer (15 minutes at room temperature; 160 mM NH₄Cl, 10 mM Tris-HCl). Lysed red blood cells were subsequently discarded through washing in phosphate-buffered saline (300 x g).

Surface stainings

Next, the freshly isolated PBMCs (Peripheral Blood Mononuclear Cells) were blocked for unspecific Fc-receptor interactions by incubation in phosphate-buffered saline supplemented with 10% human serum (10 minutes at room temperature; Sigma), followed by surface staining using combinations of fluorochrome-labelled antibodies. Isotype- and fluorochrome matched control antibodies were used to define marker positivity. FACS antibody panels are outlined in Supplemental Table 3.

FACS analysis

FACS analyses were performed on an LSRFortessa cytometer (BD Biosciences). During the course of the study, the flow cytometer was daily calibrated using BD Cytometer Setup and Tracking (CST) beads, according to the instructions of the manufacturer (BD Biosciences).

Gating and data generation

The raw data files from FACS analyses were imported into FlowJo software (Treestar Inc.) which was used for all data generation. Our gating strategy is outlined in Supplemental Figure 1. In order to robustly measure chemokine receptor expression and minimize the influence of human error on the data, we chose to measure the median fluorescence intensity (MFI) of the respective CCR on the entire parent leukocyte population (i.e. CD3+ for T-cells; CD19+ for B-cells; CD14+ for monocytes; and CD16+ for granulocytes) rather than defining CCRx-positive and –negative sub-populations by the means of manual gating. Each MFI value was
subsequently normalized to the background staining of its corresponding isotype control antibody (Supplemental Figure 1).

**Statistical rationale**

**Initial variable reduction**

In order to reduce the number of analytical variables and remove chemokine receptors that did not have predictive value, a statistical exclusion criteria was set. If the isotype-control normalized chemokine receptor expression MFI was <300 in both disease groups (UC and CD), it was deemed too low to enable predictive distinction. This is due to roughness of the measuring instrument with regard to its limitation in distinguishing dim expression from negativity. Based on this criteria, 52 chemokine receptors were excluded from the analysis (Table 1, highlighted in red).

**Uni-variate logistic regression**

In order to create a predictive model, the logistic regression method was then utilized. Thus, 28 univariate logistic regression analysis were performed on each remaining chemokine receptor to identify the receptors that were most likely to be of predictive value. Since the main aim of this analysis was to identify potential predictive chemokines that could be further studied in an extended cohort, type I errors were deemed more feasible to conduct compared to type II. Based on this rationale, alpha \((\alpha)\) was set to 0.1.

**Multiple logistic regression and predictive model generation**

The 28 univariate logistic regression revealed four chemokine receptors that had a corresponding p-value below 0.1; that was CXCR4\(^{CD3}\), CXCR1\(^{CD16}\), CCR9\(^{CD14}\) and CCR4\(^{CD14}\). Those four chemokines were they put into a multiple logistic regression which generated the following model (where values closer to 0 = CD; and values closer to 1 = UC):

\[
\text{Logistic regression model: }
1 = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \epsilon
\]

\[
\text{where } x_1 = \text{CXCR4}^{CD3}, x_2 = \text{CXCR1}^{CD16}, x_3 = \text{CCR9}^{CD14}, x_4 = \text{CCR4}^{CD14}, \text{and } \epsilon \text{ is the error term.}
\]
In the multiple logistic regression, two UC patients were excluded from the analysis due to missing data in the CD16/CXCR1 variable. The likelihood ratio test revealed that the model had a p-value of $p=0.0033$. The model managed to separate the data correctly in 92.31% of the cases in the dataset.

References

### Supplemental Table 1

#### Ulcerative colitis (n=16)

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<thead>
<tr>
<th>Sex (girls, boys)</th>
<th>7/9</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>14.5 (2.5) (8.8-17.4)</td>
</tr>
<tr>
<td>Symptom duration</td>
<td>3.2 (3.2) (1.5-18.0)</td>
</tr>
</tbody>
</table>

#### Paris extension:

- E1: 2
- E2: 0
- E3: 2
- E4: 12

#### PUCAI, median (IQR) range

- 40 (31.3-53.8) 20-70

#### Crohn's disease (n=12)

<table>
<thead>
<tr>
<th>Sex (female/male)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>12.3 (9.8-13.5) (7.1-15.5)</td>
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<tr>
<td>Symptom duration</td>
<td>5.8 (3.1-10.9) (1.0-12.0)</td>
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</tbody>
</table>

#### Paris extension:

- A1aL1B1G1: 1
- A1bL1B1G0: 2
- A1bL2B1G0: 2
- A1bL1B1G0: 1
- A1bL2L4bB3G1: 1
- A1bL3L4aB1G0: 1
- A1bL2L4aB1G0: 1
- A1bL3L4aB1G0p: 1

#### PCDAI

- 26.5 (20-36.6) 20-42.5

#### Healthy controls (n=17)

<table>
<thead>
<tr>
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<td>Age (years)</td>
<td>9.6 (4.6-14.5) (1.5-17.0)</td>
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## Supplemental Table 2

<table>
<thead>
<tr>
<th>Diagnostic characteristics</th>
<th>Crohn’s disease (n=12)</th>
<th>Ulcerative colitis (n=16)</th>
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<td>Ileocolonoscopy</td>
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<td>Colonic disease</td>
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<td>ESR ≥10</td>
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<td>≥30</td>
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<td>F-Calprotectin ≥500-1499</td>
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<td>1500-8500</td>
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<tr>
<td>Histopathological changes of CD (granulomas, transmural disease, crypt abscesses, giant cells, ileitis etc.)</td>
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<tr>
<td>Histopathological changes of UC (basal plasmocytosis, crypt abscesses, crypt ulcerations etc.)</td>
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### Supplemental Table 3. Flow cytometry antibody conjugates used in the study.

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Exclusive enteral nutrition: clinical effects and changes in mucosal cytokine profile in children with first onset inflammatory bowel disease

Background: Exclusive Enteral Nutrition (EEN) is first line treatment in children with Crohn’s disease (CD) for induction of remission. Still, the mode of action remains conjectural. The aim of this study was to investigate whether the effect of EEN as induction therapy is paralleled by changes in the colonic mucosal cytokine profiles (CMCP).

Methods: Twelve children with new onset IBD were investigated per the ECCO/ESPGHAN protocol. They received six weeks of EEN (polymeric), and a control colonoscopy was performed shortly after completion of treatment. The patients were assessed clinically, and endoscopic and histologic scoring were obtained. Fourteen cytokines were measured by quantitative real-time polymerase chain reaction (PCR) at onset and at control endoscopy.

Results: Twelve children (six girls) with a median age of 12.5 years and a median duration of symptoms at diagnosis of 5.8 month, completed six weeks of EEN, except from one child who completed 4 weeks. At control colonoscopy, significant reductions were seen in SES-CD and 83 % were in complete clinical remission. Decreases as well as increases were seen in individual patient’s CMCP, and at diagnosis significantly higher values were measured in IL-12p (p=0.008) and IL-23a (p=0.02) in the IBD patients compared to non-IBD controls.

Conclusion: In this small study EEN for induction of remission was highly effective. An overall decrease in pro-inflammatory cytokines were seen after treatment but without reaching statistical significance. Further studies are warranted to understand the role of CMCP in EEN.

Helena Rolandsdotter, MD, Kerstin Jönsson-Videsäter PhD, Ulrika L. Fagerberg, MD, PhD, Michael Eberhardson, MD, PhD and Yigael Finkel, MD, PhD
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FUNDING: This study has been supported with an unrestricted research grant from Nutricia Sweden, and with a research grant from the Kempe-Carlgrenska research foundation and from their research foundation.

Background

Crohn’s disease (CD) and Ulcerative Colitis (UC), the major entities of inflammatory bowel disease (IBD), are chronic inflammatory conditions of the gastrointestinal tract. Abdominal pain, nausea, and bloody diarrheas are common symptoms in both children and adults, and growth retardation is frequently seen in pediatric patients. Childhood IBD is characterized by more severe symptoms and wider disease extension at diagnosis than adults. Genetic, environmental, microbial and immunological factors all contribute to the development of CD, but the etiology is not yet fully understood. Exclusive Enteral Nutrition (EEN) is first-line remission treatment in Europe for CD in pediatric patients, with an overall clinical remission rate of 73-80% and it effectively induces mucosal healing (MH), superior than corticosteroids. EEN consists of liquid formulas, either elemental (formulations of amino acids), semi-elemental (formulations of amino acids and oligopeptides), or polymeric (whole protein formulas). Children who suffer from weight loss and growth retardation benefit from the additional nutritional advantage of this high energy nutrition that is not associated with physical side-effects. EEN is recommended to all children with luminal disease, including those with colonic involvement. The immunological effects driven by EEN that contribute to mucosal healing are not yet fully understood.

The intestine hosts the majority of the body’s immune cells (70-80%). The interaction between a disrupted microbial composition, an impaired intestinal mucosal barrier and the mucosal immune system is considered to play an important role in the IBD development and its chronicity. In addition, a deficiency of host immunity may also contribute to the pathogenesis of IBD. The mucosal immune cells; macrophages, monocytes, T-cells and innate lymphoid cells produce as well as are orchestrated by cytokines; small immune-regulating messenger proteins. The cytokines play a crucial part in the inflammatory response by regulating cell-differentiation, producing pro-inflammatory cytokine and recruiting white blood cells from the bone marrow. After innate immune activation, the inflammation is suggested to be mediated principally by T-cells directed by an imbalance between pro-and anti-inflammatory cytokines. The cytokine profiles in the intestinal mucosa in children with CD are fairly unknown and the cytokine landscape is still not explored. Recent reports show that temporal aspects may also be of great importance; the same cytokine may be protective during acute inflammation but harmful and perpetuate the chronic inflammation.

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Aims

To investigate the clinical effect of EEN as induction therapy, and whether the effect of EEN is paralleled by changes in the colonic mucosal cytokine profiles (CMCP). For this purpose, we used a real-time PCR with a selection of 14 pro- and anti-inflammatory cytokines as well as regulatory cytokines in treatment-naïve children with first onset of CD.

Children and methods

This is a prospective cohort study of 19 children with new onset symptoms of possible IBD between August 2013 and September 2016. Thirteen children who fulfilled the ECCO/ESPGHAN consensus criteria for pediatric CD entered the study. Five patients did not receive any IBD diagnosis and one was found to have a juvenile polyp. These six patients are further referred to as the non-IBD controls. The inclusion criteria for this study were previously healthy children up to 18 years of age, and the exclusion criteria were any use of immunosuppressive drugs within 6 months prior to inclusion. Table 1.

Examinations

All patients were investigated according to the ECCO/ESPGHAN guidelines for pediatric IBD with initial laboratory investigations: peripheral blood count, liver enzymes, albumin, erythrocyte sedimentation rate (ESR), iron status, C-reactive protein (CRP), fecal calprotectin, as well as stool cultures to exclude infectious diarrhea. At inclusion and after complete EEN treatment, all patients were measured for weight (in cm) and height (in kg minus 700 grams for the estimated weight of clothing).

Endoscopy

In line with the ECCO/ESPGHAN guidelines for new onset IBD in children, all patients were investigated with upper endoscopy and ileocolonoscopy under general anesthesia with multiple mucosal biopsies for histopathological evaluation from esophagus, corpus, antrum and from proximal and descending duodenum, ileum, cecum, ascending, transverse, descending and sigmoid colon and rectum. For study purposes, three biopsies were taken from corpus and three from antrum in the ventricle, and four colonic biopsies from the most inflamed site and four biopsies from the least inflamed site in the colon. The endoscopy examinations were documented by a still picture system integrated in the computerized medical record program (Picsara, Mawell, Solna, Sweden). The mucosal biopsies were reviewed by an experienced pathologist using the Geboes scoring system after completion of the study. A control upper and lower endoscopy with multiple biopsy harvesting following the same protocol as for the diagnostic endoscopy, was performed shortly after completion of EEN remission treatment in children with CD. No control colonoscopy was performed in the six non-IBD controls.
Clinical assessment of disease activity

We used the validated disease activity scoring PCDAI (Pediatric CD Activity Index) that encompasses abdominal pain, number of liquid stools, general wellbeing, abdominal examination, perirectal disease, extraintestinal manifestations, weight, height, hematocrit, albumin and ESR. A <10 points for remission, 10–27.5 for mild disease, >27.5–37.5 moderate disease, and >37.5–100 for severe disease. A PCDAI increase of >12.5 points reflects a clinically significant response to treatment. PCDAI score was assessed at inclusion and at control colonoscopy.

Assessment of disease extension and mucosal healing

Disease extension was assessed by Paris classification. We used Short Endoscopic Scoring Crohns Disease (SES-CD) to assess the mucosal inflammation in the CD patients. It was evaluated either by the endoscopist or by the researcher from the charts (the endoscopy history and endoscopy imaging). SES-CD describes the presence and size of ulcers, extent of the ulcerated surface, extent of the affected surface, and the presence and type of narrowing scored with 0-3 points in rectum, sigmoid and left colon, transverse colon, right colon and ileum. Remission is equal to 0-2 points, mild disease as 3-6 points, moderate 7-15 points and severe disease ≥16 points. The mucosal biopsies were reviewed by an experienced pathologist using the Geboes scoring system which is a validated histological score in IBD. Grades <3 were regarded as absence of IBD inflammation, while grades ≥3 were regarded as presence of IBD inflammation of varying degree (1-3:1-3:7) with the following subgroups: a=basal plasmocytosis, b=lymphoid nodules, c=paneth cell hyperplasia, d= eosinophils in the lamina propria, e=histiocytic cell proliferation, f=giant cells, g=granuloma, h=vasculitis and i=dysplasia.

Exclusive Enteral Nutrition (EEN)

We used Fortimel Energy®, a polymeric formula, which was prescribed according to daily energy intake estimated at 120 % of recommended daily allowance (RDA). Fortimel Energy® contains 625 kJ/100 ml, 5.8 g fat and 5.9 g protein/100 ml. The EEN treatment was introduced by daily step-wise increase by 25% of full daily intake during three days followed by six weeks of EEN and rounded off by step-wise decrease in by 25% of full daily intake for 3 days. At de-escalation the patients were advised to avoid large amounts of dairy products, spicy food and large amount of legumes. The children were allowed to drink clear beverages, eat popsicles and eat a few hard mints per day during the EEN treatment. In case the patients were not satiated, they were allowed additional nutritional drinks until satisfied. The children and care-givers had one hourly introductory session with the dietitian (J.R.). Thereafter, the dietitian monitored the patients and caregivers weekly with motivating phone calls. Scheduled follow-ups with clinical assessments were arranged at week 2 of EEN.

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Cytokine-selection

A panel of cytokines known to participate in chronic intestinal inflammation was selected for testing. We investigated CSF-2, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, IL-12β, IL-13, IL-22, IL-23α, IL-36γ, TGF-β1 and a control gene ABL. The amount of mRNA in the 14 different pro-inflammatory mediators was compared to the signal from the ABL-gene and the result is presented as the ratio cytokine/ABL. We used TaqMan® Gene Expression Assays, applied biosystems (Thermo Fischer Scientific®, Waltham, MA, USA)

RNA extraction and gene expression by quantitative real-time PCR

Biopsies for CMCP were put into RNA-later (Invitrogen, Waltham, MA USA) and kept at +6°C for 24 hours and then frozen (-20°C) until analysis. Total RNA was isolated utilizing the Fibrous tissue kit (Qiagen, Hilden, Germany) with slight modification. Defrosted and minced biopsies (3mm) were homogenized with a pestle motor (WVR®, Radnor, PA USA) for 30-60 seconds in 350 µL RLT-buffer and 350 µL 70% ethanol was added. The homogenates were loaded to spin columns, centrifuged and the columns were washed with 350 µL RW1-buffer. Samples in columns were treated by DNase I and washed with 350 µL RW1-buffer, the remainder handled according to the manufacturer protocol. cDNA was obtained by reverse transcription. Quantitative real-time PCR (qPCR) was performed using the 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) for quantification. Probes were obtained from Applied Biosystems, TaqMan® MGB probes, FAM™ dye-labeled; IL-1 (Hs00174097_m1), IL-4 (Hs00174122_m1), IL-5 (Hs01548712_g1), IL-6 (Hs00985639_m1), IL-10 (Hs00961622_m1), IL-12 (Hs01015158_m1), IL-13 (Hs00174379_m1), IL-22 (Hs0154134_m1), IL-23 (Hs00900828_g1), IL-36 (Hs00219742_m1), IL-10 (Hs00998133_m1), GM-CSF (Hs00929873_m1), ABL1 (Hs01104728_m1) according to manufacturer protocol. Fold increases of mRNA transcripts were calculated as follows: ΔΔCt = Ct (gene of interest) – Ct (ABL1), ΔΔCt = ΔCt sample – average ΔCt control group, and fold difference = 2−ΔΔCt. For technical reasons, CMCP was not analyzed in all children with IBD both at diagnosis and at colonoscopy. No active selection was performed.

Ethical approval

The study was approved by the local Ethics Committee in Stockholm, Sweden. (No. 2010/1252-31/). Informed written consent was obtained from legal guardians for patients and also from all patients over 15 years of age before any study-related procedure was initiated in accordance with the Helsinki II Declaration.

Statistics

Comparisons between clinical chemistry values, SES-CD, and disease activity index (PCDAI) before and after treatment, were conducted with paired sample t-tests (parametric data) or Wilcoxon paired t-test (non-parametric data) depending on the normal distribution which

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RNA extraction and gene expression by quantitative real-time PCR

Biopsies for CMCP were put into RNA-later (Invitrogen, Waltham, MA USA) and kept at +6°C for 24 hours and then frozen (-20°C) until analysis. Total RNA was isolated utilizing the Fibrous tissue kit (Qiagen, Hilden, Germany) with slight modification. Defrosted and minced biopsies (3mm) were homogenized with a pestle motor (WVR®, Radnor, PA USA) for 30-60 seconds in 350 µL RLT-buffer and 350 µL 70% ethanol was added. The homogenates were loaded to spin columns, centrifuged and the columns were washed with 350 µL RW1-buffer. Samples in columns were treated by DNase I and washed with 350 µL RW1-buffer, the remainder handled according to the manufacturer protocol. cDNA was obtained by reverse transcription. Quantitative real-time PCR (qPCR) was performed using the 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) for quantification. Probes were obtained from Applied Biosystems, TaqMan® MGB probes, FAM™ dye-labeled; IL-1 (Hs00174097_m1), IL-4 (Hs00174122_m1), IL-5 (Hs01548712_g1), IL-6 (Hs00985639_m1), IL-10 (Hs00961622_m1), IL-12 (Hs01015158_m1), IL-13 (Hs00174379_m1), IL-22 (Hs0154134_m1), IL-23 (Hs00900828_g1), IL-36 (Hs00219742_m1), TGF-β (Hs00998133_m1), TNF-α (Hs01113624_g1), IFN-γ (Hs00989291_m1), GM-CSF (Hs00929873_m1), ABL1 (Hs01104728_m1) according to manufacturer protocol. Fold increases of mRNA transcripts were calculated as follows: ΔΔCt = Ct (gene of interest) – Ct (ABL1), ΔΔCt = ΔCt sample – average ΔCt control group, and fold difference = 2−ΔΔCt. For technical reasons, CMCP was not analyzed in all children with IBD both at diagnosis and at colonoscopy. No active selection was performed.

Ethical approval

The study was approved by the local Ethics Committee in Stockholm, Sweden. (No. 2010/1252-31/). Informed written consent was obtained from legal guardians for patients and also from all patients over 15 years of age before any study-related procedure was initiated in accordance with the Helsinki II Declaration.

Statistics

Comparisons between clinical chemistry values, SES-CD, and disease activity index (PCDAI) before and after treatment, were conducted with paired sample t-tests (parametric data) or Wilcoxon paired t-test (non-parametric data) depending on the normal distribution which
was tested with Shapiro-Wilks test. Paired comparisons between cytokines before and after treatment were conducted with Wilcoxon paired t-test and comparisons between groups were conducted with Mann-Whitney U test after control of normality with Shapiro-Wilks test. All analyses were performed with the IBM SPSS Statistics Data Editor, version 23. Statistical significance was set at p<0.05.

RESULTS

EEN treated patients

Thirteen children received a CD diagnosis according to the EECO/ESPGHAN guidelines. One patient with celiac disease was on strict gluten-free diet at diagnosis, the other twelve patients were previously healthy before IBD onset. Familiar occurrence of IBD was noted in six children, four with one first-degree relative and two with one second-degree relative. One CD patient left the study after the first colonoscopy due to social circumstances. After EEN treatment completion, two patients were re-diagnosed with UC and one with IBD-U. Table 1 for Paris classification at inclusion.

Enteral Nutrition

Eleven children completed six weeks of EEN with three days of escalation and three days of de-escalation of the nutritional drinks, one CD patient completed only four weeks of EEN due to lack of motivation, but fulfilled participation in all other aspects of the study. All patients accepted the prescribed EEN without the use of nasogastric tube. The treatment was overall tolerated without side effects in all 12 patients. The dietician guided the patients through the treatment with weekly motivating phone calls, both with the caregiver and the patients. Most patients preferred to drink one or two flavors during the whole EEN period. During the two first weeks of treatment, two patients suffered from mild stomach pain during intake of the nutritional drinks, which could be ignored.

Disease activity

Clinical activity scoring

All patients clinical scoring was measured by PCDAI including the children who later changed to UC and IBD-U. At inclusion, six children were considered to have mild disease (PCDAI ≤10 to ≤ 27.5) while six patients showed moderate to severe disease (PCDAI ≥ 27.5). There was a significant decrease in PCDAI at control colonoscopy (p<0.02) with median PCDAI 26.5 (IQR 20.0-36.6, range 20-40) at inclusion and median PCDAI 5 (IQR 0-5, range 0-15) after EEN treatment. Ten of twelve patients (83%) showed clinical remission (PCDAI <10) after the induction treatment. One child received concomitant medication with mesalazine (53 mg/kg/day) due to clinical disease activity.

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Laboratory values
ESR, CRP, hemoglobin, albumin and F-calprotectin were monitored. There were significant decreases in ESR (p=0.005), CRP (p=0.016) and F-calprotectin (p=0.033) and significantly raised hemoglobin (p=0.001) and albumin (p=0.003) after EEN treatment (Table 2).

Anthropometric data
There was a significant gain in weight (p=0.003) and height (p=0.027) (Table 2).

Endoscopic healing
All patients were examined with control upper and lower endoscopy at median 13 days (range 1-45 days) after completion of EEN. A significant decrease (p=0.008) was seen in SES-CD after complete EEN (median SES-CD 9.5, IQR 4.5-14.3, range 4-28 at inclusion, and median 3.5, IQR 1-10, range 0-16 at control colonoscopy).

Clinical outcome of the two UC patients
One UC patient showed decreased PCDAI from 23 to 5 points and ESR from 28 to 11 mm/h, and a slight raised Hb (110-116 g/L). However, no effect was seen in SES-CD, CRP, and F-calpro The other UC patient showed a decrease in PCDAI (40 to 15 points), ESR (53-2 mm/h), CRP (47-11 mg/L), F-calprotectin (3169-1430 mg/kg) and SES-CD (21-16 points) and a raise in albumin (28-31 g/L) and Hb (83-128 g/L).

Mucosal healing
The mucosal biopsies were histologically scored using the Geboes score. This showed improvements in ten patients, worsening in one patient and no inflammation in one patient. Appendix 1.

CMCP
In six of twelve EEN treated children, the cytokine profile in biopsies were analyzed both at diagnosis and at control endoscopy. In the remaining seven patients, mucosal cytokines in biopsies were analyzed either at diagnosis or at control colonoscopy.

Cytokine profiles in six patients before and after EEN treatment
In six EEN treated patients who completed six weeks of EEN (five with CD and one with IBD-U) no significant differences were found in any cytokines. In individual patients decreases as well as increases in the cytokine expressions were seen. Figure 1. With regards to the key pro-inflammatory cytokine IL-12β, only one patient had a decrease while four patients had an increase after EEN. In addition, the anti-inflammatory IL-10 decreased in three patients and increased in three patients, while two patients had a decrease of the regulatory TGF-B1, one patient had no change and three patients showed increased expression after EEN.

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treatment. The regulatory cytokine CSF-2, were down-regulated in four out of five patients after EEN treatment (Table 3 and figure 1).

CMCP in eight patients at inclusion and seven patients after EEN treatment

We compared the CMCP in the available biopsies from inclusion (n=eight) and in the available biopsies after EEN treatment (n=seven). Non-significant decreases were seen in IL-1β (p=0.064), and IL-23α (p=0.064).

Cytokine comparison between IBD patients and non-IBD controls

When CMCP in six non-IBD patients where compared to eight IBD-patients at inclusion, significantly higher values of IL-12β (p=0.007) and IL-23α (p=0.025) were measured in the IBD patients compared to non-IBD controls.

DISCUSSION

In the present study, we wanted to investigate the clinical, endoscopic and histological remission rate and to further investigate changes in mucosal cytokine profiles in 12 children with new onset IBD who were treated with EEN as induction remission treatment.

Remission induction treatment with EEN in 12 patients with IBD

We found significant decreases of PCDAI, CRP, ESR, and F-calprotectin and a significantly increased Hb and albumin as well as a significant gain in weight and height between time of inclusion and after induction of remission with EEN treatment. Mucosal healing, measured by SES-CD, was seen at control colonoscopy compared to diagnosis. Our results are in line with other reports showing that EEN impel the patient into clinical and biochemical remission in up to 80 % in children with newly diagnosed CD.10 Stewart et al. investigated the attitudes and practice patterns of EEN use among members of North American Society of Gastroenterology, Hepatology and Nutrition, and found that difficulty maintaining compliance was the number one factor that limits the use of EEN, and further, that 71% of the doctors used nasogastric/gastric tube feeding as their usual route of administration.20

Previous reports have not shown any difference in MH between elemental, semi-elemental, or polymeric nutritional drinks.4 Thus, we used a polymeric nutritional drink; because better taste permits oral administration. EEN is used as first line treatment for children with CD, and an important dialogue is held with the patient and its caregivers of the treatment options and the beneficial quality of EEN. All patients in our study were offered a feeding tube but no patient wanted or needed one. The dietician had close contact with the patients throughout the treatment which we believe is of great importance when it comes to adherence to the therapy. Thus, our conclusion is that a polymeric drink, and tight follow-ups by the dietician is a successful way for achieving completion of the entire EEN treatment period. Interestingly, the two patients with a subsequent change of diagnosis from CD to UC
Inflammatory cytokines from dietitians

In this pilot study, no significant differences in CMCP were found after EEN treatment in children with IBD. However, when CMCP where compared to non-IBD patients significant higher levels of IL-12β and in IL-23α were measured, and these cytokines are known to play an important role in the pathogenesis of CD. Our intention was to investigate the effects of EEN on the mucosal cytokine profile in addition to the clinical effect. Probably, CMCP mirrors the inflammatory activity more adequately than cytokines analyzed in peripheral blood or feces. Even though decreases of pro-inflammatory cytokines were seen after EEN treatment, no significant fluctuations were identified due to individual variations and a limited number of patients, which is an important limitation of the study.

In the expression of the regulatory cytokines TGF-β1, CSF-2, and IL-10, both decreases and increases were noticed after treatment. There are only a few reports published on mucosal cytokines after EEN. Yamamoto et al. investigated IL-1β, IL-1 receptor antagonist (IL-1ra), IL-6, IL-8, and TNF-α with enzyme-linked immunosorbent assay (ELISA), before and after four weeks of elemental diet in 28 adult patients. After treatment, the mucosal concentrations of IL-1β, IL-2ra, IL-6, IL-8, and TNF-α normalized. Furthermore, they compared the IBD patients to healthy controls and found significantly higher levels in all cytokines in the IBD patients. Fell et al. investigated clinical efficacy and mucosal healing after eight weeks of CT3211 (a polymeric nutritional diet) in 29 children with active CD. In addition, IL-1β, IL-8, IL-10, TGF-β1 and IFN-γ were analyzed with PCR in 18 children in ileal- and colonic biopsies. Complete clinical remission was seen in 79% of the patients, and macroscopic and histological healing was associated with a significant down-regulation of mucosal pro-inflammatory cytokines in ileum or/and colon. In the ileum an increased expression of TGF-β1 was noticed. An elevation of the cytokines was also seen in the CD patients compared to non-IBD controls.

Conclusion

In our experience EEN treatment, performed with a polymeric formula and engagement from dietitians and doctors, is a successful way to achieve completion of EEN in children with CD. The mode of action of EEN is still not refuted, but an overall decrease in pro-inflammatory cytokines were seen. Nevertheless, a better understanding of temporal also showed improvement after EEN treatment, though evaluation of EEN in UC in earlier reports is difficult to find.

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### Table 1. Demography and Paris classification of 13 EEN treated patients at inclusion

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<thead>
<tr>
<th>Sex (girls/boys)</th>
<th>Age (in years, median IQR range)</th>
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<td>9/7</td>
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<td>A1bL3L4aB1G0p</td>
<td>152 (148.5-159) 69.4 (62.5-76.5)</td>
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<td>143 (134-152) 62 (53-70)</td>
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### Table 2. Laboratoy values, weight and height at inclusion and at control colonoscopy

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Figure 1. Cytokine profiles in mucosal biopsies according to mRNA levels measured by qRT-PCR before and after EEN treatment.

1A. CSF-2 at onset and after EEN treatment
1B. IFN-γ at onset and after EEN treatment
1C. TNF-α at onset and after EEN treatment
1D. IL-10 at onset and after EEN treatment
1E. IL-12β at onset and after EEN treatment
1F. IL-23α at onset and after EEN treatment


APPENDIX 1

**Geboes scoring:** Grades <3 were regarded as absence of IBD inflammation, while grades ≥3 were regarded as presence of IBD inflammation of varying degree (3:1:3:7) with the following subgroups: a=basal plasmocytosis, b=lymphoid nodules, c=paneth cell hyperplasia, d= eosinophils in the lamina propria, e=histiocytic cell proliferation, f=frgiant cells, g=granuloma, h=vasculitis and i=dysplasia.

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Geboes results summarized (every patient, 11 segments), as improvement, no change, aggravated inflammation or no inflammation.
**ARTICLE**

**Mucosal Cytokine Profiles After Induction Therapy With Granulocyte/Monocyte Apheresis in New-onset Inflammatory Colitis**

*Helena Rolandsdotter,* *Kerstin Jönsson-Videfelt,* *Ulf E. Fagerberg,* *Michael Eberhardson,* and *Yigael Finkel*

**ABSTRACT**

Granulocyte/monocyte apheresis (GMA) selectively removes circulating granulocytes and monocytes; important producers of proinflammatory cytokines. New-onset inflammatory bowel disease (IBD) is a disease of unclear etiology, with two major subtypes: ulcerative colitis (UC) and Crohn’s disease (CD). Little information about the impact of GMA on the mucosal inflammatory environment in children with IBD is available. We investigated the colonic mucosal cytokine profiles (CMCP) in children with new-onset IBD colitis (7). In this study we report the colonic mucosal cytokine profiles in children with new-onset inflammatory bowel disease (IBD) colitis.

**What Is Known**

- Granulocyte/monocyte apheresis (GMA) selectively removes circulating granulocytes and monocytes; important producers of proinflammatory cytokines.
- New-onset IBD is a disease of unclear etiology, with two major subtypes: UC and CD.
- Little information about the impact of GMA on the mucosal inflammatory environment in children with IBD is available.

**What Is New**

- We analyzed CMCP in 7 children with new-onset IBD colitis included in a previous study (7) and in 6 children who underwent endoscopy for unexplained gastrointestinal disease. 2 of these were healthy, and 1 patient had a single jejunal polyp. These children are referred to as non-IBD controls. The patients with IBD are referred to as non-IBD controls. The patients with IBD fulfilled the 2017 ESPGAN criteria for IBD.

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used TaqMan Gene Expression Assays, Applied Biosystems control gene ABL CT. The amount of mRNA in the 14 different children, 12 to 18 years of age, with a body weight (TNF) in human blood (2,13). The granulocyte adsorption ability of the column and returns to the cubital vein in the contralateral arm. Blood is circulated through the column at a flow rate of 30 mL/min thought to be the proinflammatory CD14 receptors of the activated granulocytes and monocytes (Paris classification) (8) and Mayo endoscopic scoring (10) in the older than 15 years before any study-related procedure was initiated. The study was approved by the local Ethics Committee in Stockholm, Sweden (no. 2011/1927–31/2). Informed written consent was obtained from legal guardians and also from all children older than 15 years before any study-related procedure was initiated, in accordance with the Helsinki II Declaration.

**Statistics**

Comparisons between clinical chemistry test, Mayo endoscopic score and PCDAI before and after treatment, were conducted with paired sample t-tests (parametric data) or Wilcoxon signed-rank test (nonparametric data). Comparisons between the period of diagnosis and treatment (Mann-Whitney test) and between cytokines in the peripheral blood and mucosa (t-test) were performed. All analyses were performed with the IBM SPSS Statistics (for nonparametric) befor...
Clinical Outcome and Colonic Mucosal Cytokine Profiles in 7 Children at Inflammatory Bowel Disease Onset and After Induction Treatment

Clinical and Endoscopic Remission
At inclusion the median PUCAI was 90 (interquartile range [IQR]: 60–90, range: 80–100). At time for control colonoscopy there was a significant improvement in PUCAI (median 0, IQR: 0–5, range: 0–25) (P = 0.001) (Table 1). The pediatric Mayo scoring showed a significant improvement in clinical score (median 2, IQR: 2–5, range: 2–5) and control colonoscopy (median 1, IQR: 1–2, range: 1–2) (P = 0.002). An endoscopic remission was found in none of the biopsies from 4 patients, in 10% to 30% of the biopsies from 3 patients. Accidental colonic biopsies (Goblet score >3) was seen in none of the biopsies in 1 patient, in 25% to 40% of the biopsies from 3 patients, in 70% to 75% of the biopsies from 2 patients, and in all biopsies from 1 patient.

Colonic Cytokine Comparison Between Inflammatory Bowel Disease Colitis Patients and Noninflammatory Bowel Disease Controls
In 8 non-IBD controls, CMCP showed significantly lower IL-12-α (P = 0.025) and R-E22-α (P = 0.040) compared to the 7 post-treatment biopsies from the 3 patients. IL-36α (P = 0.82) was also shown in the non-IBD patients without reaching statistical significance.

Colonic Mucosal Cytokine Profiles in 2 Children With Crohn's Disease
Post-treatment cytokine expression in the 2 CD colitis patients after induction treatment was overall decreased except for an increase in IL-22 and IL-10.

Colonic Mucosal Cytokine Profiles in 1 Child Treated With Prednisolone
The child did not achieve remission, yet CRP (P = 0.018), TNF-α (P = 0.80), IL-23α (P = 0.84), IL-18 (P = 0.02), IL-19 (P = 0.03), IL-22 (P = 0.02), and TGF-β (P = 0.045) in the irritable mucosa after induction treatment (IL-4 (P = 0.006), IL-5 (P = 0.006), IL-6 (P = 0.071) showed a decreasing trend without statistical significance. No significant difference were seen in TNF-α (P = 0.71), IL-12-α (P = 0.499), IL-22 (P = 0.590), and IL-19 (P = 0.114).

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compared CMCP from the diagnostic endoscopy between the IBD patients and non-IBD control. We found clinical and endoscopic improvements as well as significant decreases in mucosal cytokines at control colonoscopy. Fourteen different cytokines were measured and significant decreases were seen in CMCP 2, TNF-α, IL-1β, IL-6, IL-10, and IL-12p70 in the patients with IBD compared to the non-IBD controls. These findings are in line with our findings of significantly decreased endoscopic Mayo score, and improved histological scoring, with a decrease of the neutrophil infiltration in the epithelium in a majority of our study group.

In conclusion, we studied the clinical and endoscopic remission and mucosal cytokine profiles after GMA in 28 UC adults with moderately active disease. Our findings corroborate their results from studies in adults and show that GMA decreases the endoscopic Mayo score, and improved histological scoring, with a decrease of the neutrophil infiltration in the epithelium in a majority of our study group.

We also found significantly higher IL-1α, IL-1β, IL-6, and IL-10 in 7 children at diagnosis and control endoscopy after GMA compared to the non-IBD children. Our findings are in line with our findings of significantly decreased endoscopic Mayo score, and improved histological scoring, with a decrease of the neutrophil infiltration in the epithelium in a majority of our study group.

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FIGURE 1. A-D, Murine mIFNα expression of TNF-α, IL-1β, TGF-β1, and IL-10 in 7 children at diagnosis and control endoscopy after GMA and mucosal induction. The values are presented as cytokine/ABL ratio. CD – Crohn disease, I – immunoblock; P – prednisolone treated; TGF-β1 – transforming growth factor; TNF-α – tumor necrosis factor.

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5-aminosaliclic acid to restore immune regulation in patients with IBD. Linton et al (19) showed increased levels of CD40-β-HLA-DR by circulating monocytes, that produce high levels of inflammatory mediators in patients with active IBD compared to healthy controls. After treatment with GMA and mesalazine induction, the proportion of circulating CD40-β-HLA-DR+ monocytes was significantly reduced which may explain the reductions in mucosal cytokine expression. Tanaka et al (20) showed that responders to GMA had restoration of mucosal erosions and ulcers and a marked reduction of infiltrating leukocytes, and further that mononuclear patients with short duration of UC were the best responders. Their findings are in line with our findings of significantly decreased endoscopic Mayo score, and improved histological scoring, with a decrease of the neutrophil infiltration in the epithelium in a majority of our study group.

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