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

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

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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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.

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 om 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 1). 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.

LIST OF SCIENTIFIC PAPERS

I **Rolandsdotter H**, Marits P, Sundin U, Wikström A-C, Fagerberg UL, Finkel Y, Eberhardson M.
Serum-Infliximab Trough Levels in 45 Children with Inflammatory Bowel Disease on Maintenance Treatment. International Journal of Molecular Sciences. 2017 Mar 7;18(3). pii: E575. doi: 10.3390/ijms18030575.

II **Rolandsdotter H**, Eberhardson M, Fagerberg UL, Finkel Y.
Granulocyte and Monocyte Apheresis for Induction of Remission in Children with New-Onset Inflammatory Bowel Colitis. J Pediatr Gastroenterol Nutr. 2017 Jun 9. doi: 10.1097/MPG.0000000000001641

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.
Mucosal cytokine profiles after induction therapy with granulocyte/monocyte apheresis in new onset inflammatory colitis. J Pediatr Gastroenterol Nutr. 2017 Sep 7. doi:10.1097/MPG.0000000000001735

INNEHÅLL

LIST OF ABBREVIATIONS

5-ASA	5-Aminosalicylates
ADA	Adacolumn®
ALP	Alkaline phosphatase
ATI	Antibodies toward infliximab
AZA	Azathioprine
CC	Control colonoscopy
CCR	Chemokine receptor
CD	Crohn's disease
CDAI	Crohn's disease activity index
CDEIS	Crohn's disease endoscopic index of severity
CMCP	Colonic mucosal cytokine pattern
CRP	C-Reactive protein
CS	Corticosteroids
DC	Dendritic cells
EEN	Exclusive enteral nutrition
ESPGHAN	European Society of Gastroenterology, Hepatology Nutrition
ESR	Erytrocyte sedimentation rate
F	Fecal
FCP	Fecal calprotectin
GI	Gastrointestinal
GMA	Granulocyte monocyte apheresis
GSF	Granulocyte colony stimulating factor
IBD	Inflammatory bowel disease
IBD-U	IBD unclassified
IEC	intestinal epithelial cell

IFN	Interferon
IFX	Infliximab
IL	Interlukein
LOR	Loss of response
NK-cells	Natural killer cells
PBS	Phosphate buffered saline
PCDAI	Pediatric Cronh's Disease Activity Index
PRR	Pattern recognition receptors
PSC	Primary xclerosing cholangitis
RT	Room temperature
SD	Standard deviation
TDM	Therapeutic drug monitoring
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UC	Ulcerative colitis
PRR	Pattern recognition receptor
PUCAI	Pediatric Ulcerative Colitis Activity Index
SES-CD	Simple endoscopic score for Crohn's disease

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[7, 8].

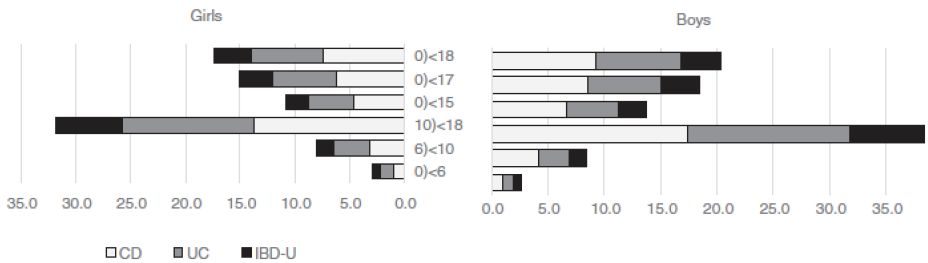
1.2 EPIDEMIOLOGY

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[9]. 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. Furthermore, the incidence rates for women were significantly higher in both UC and CD than for men[10]. Today, the overall prevalence of IBD in Sweden is approximately 0.65%[11].

Approximately 10-20% of all IBD cases are diagnosed during childhood[12] 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)[13-15]. The incidence of pediatric IBD has increased sharply in recent decades, as is true for the whole Northern hemisphere[11, 16-18].

In Sweden, prevalence of pediatric IBD in 2010 was 75/100,000 (0.75 ‰ of all children in Sweden in 2010) [19]. Figure 1 depicts the incidence rate from 2003 to 2013 by type of IBD, sex and age of onset[20].



		CD	UC	IBD-U	All IBD
GIRLS	0<6	1.0	1.2	0.7	2.9
	6<10	3.2	3.2	1.6	8.0
	10<18	13.7	12.0	6.0	31.8
	0<15	4.7	4.1	2.1	10.9
	0<17	6.2	5.8	3.0	15.0
	0<18	7.4	6.6	3.4	17.4

		CD	UC	IBD-U	All IBD
BOYS	0<6	1.0	0.9	0.7	2.6
	6<10	4.2	2.7	1.5	8.4
	10<18	17.4	14.5	6.6	38.4
	0<15	6.7	4.5	2.5	13.7
	0<17	8.6	6.5	3.3	18.3
	0<18	9.2	7.6	3.6	20.4

Figure 1 | Incidence rate of paediatric 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 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[21].

1.3.1.1 Genetics in IBD

201 susceptibility loci for IBD, mostly shared between CD and UC have hitherto been discovered[22]. However, many IBD loci are also implicated in other autoimmune-mediated disorders, most notably with ankylosing spondylitis and psoriasis [23]. 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[24]. 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[25, 26]. 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[27].

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[28].

1.3.1.2 Environmental factors

Smoking and GI infections have the strongest[29] relationship to environmental factors that may trigger the onset of IBD[30, 31]. 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[32]. 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[33-35]. Infections with Clostridium difficile, a gram-positive, anaerobic, spore-forming bacillus, may also worsen the course of IBD[36].

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

cleaner water. All these factors may contribute but none have shown, either alone or in combination, any convincing association with the augmented incidence of IBD[30, 31].

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[30, 31, 37]. The innate immune responses include monocytes, macrophages and dendritic cells (DCs) that increase in correlation with disease activity[38]. 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[30, 31].

1.4 PEDIATRIC IBD

Children and youth often have more severe and more extensive disease than adults, with vast involvement and rapid early progression[39 40, 41]. 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[39].

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, malabsortion due to an inflamed mucosa, ongoing inflammation followed by an increased nutritional need, and corticosteroid (CS) treatment that interferes with growth hormones[42]. 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[43].

1.5 DIAGNOSTIC WORK-UP

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

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[44]. 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[41, 44].

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

CD Endoscopic characteristics	UC Endoscopic characteristics
Ulcers	Ulcers
Cobblestoning	Erythema
Skip lesions	Loss of vascular pattern
Strictures	Granularity
Fistulas	Friability
Segmental distribution	Spontaneous bleeding
Fissures, fistulas, abscesses	Pseudopolyps
	Continuous (proximal extension from rectum)

Table 2. Histologic characteristics of CD and UC

CD Histological characteristics	UC Histological characteristics
Submucosal or transmural involvement	Mucosal involvement
Ulcers, crypt distortion	Crypt distortion
Crypt abscesses	Crypt abscesses
Granulomas	Goblet cell depletion
Focal changes (within biopsy)	Continuous distribution
Patchy distribution (between biopsies)	

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[45]. 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[46]. 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[46].

The Pediatric UC Activity Index (PUCAI) is widely used to score patients in the everyday clinical setting, as well as for research purposes[47]. 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[47].

Other clinical CD scores currently used in adult gastroenterology are the CD Activity Index (CDAI)[48] and the Harvey and Bradshaw's Activity Index (HBI), which is a simpler version of the CDAI[49]. In UC, the Modified Truelove and Witt's Severity Index[50] and the Mayo score have been frequently used (Mayo including endoscopic findings and partial Mayo without endoscopic findings)[51].

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.

Ulcerative colitis	E1	E2	E3	E4
Location	Ulcerative proctitis	Left-sided UC, distal to splenic flexure	Extensive, hepatic flexure distally	Pan-colitis, proximal to hepatic flexure
Severity 0	Never severe	Defined as PUCAI	≥65	
Severity 1	Ever severe	Defined as PUCAI	≥ 65	

Crohn's disease				
Age at diagnosis=A	A1a 0-< 10 y	A1b 10-<17y	A2 17-40 y	A3 >40 y
Location=L	L1: distal 1/3 ileum ± limited cekal disease	L2: colonic	L3: ileocolonic	L4: isolated upper disease*
Behavior=B	B1: non-stricturing, non-penetrating	B2: structuring	B3: penetrating	B2B3: both structuring and penetrating disease
Growth=G	G1: growth delay	G0= no evidence of	growth delay	
Perianal disease=P				

*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*[52]**.**

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[53]. 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[51]. 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 scale[54].

The Mayo endoscopic score is a widely used three-point scale that has not yet been validated (Table 4)[51].

Table 4. Mayo ulcerative colitis endoscopic score.

Endoscopic Mayo score	**Endoscopic findings**
Score 0 = remission	Normal mucosa
Score 1= mild disease	Erythema, decreased vascular pattern, mild friability
Score 2 = moderate disease	Marked erythema, lack of vascular pattern, friability and erosions
Score 3 = severe disease	Spontaneous bleeding, ulceration

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 remission[55].

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 validated[56]. 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 administered[57, 58].

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.[59] 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 activity[60]. 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 evaluated[59].

Geboes score: different grades used to evaluate disease severity in UC.

Grade 0 - Structural (architectural change)
0.0 No abnormality | 0.1 Mild abnormality | 0.2 Mild/moderate diffuse or multifocal abnormalities|
0.3 Severe diffuse or multifocal abnormalities
Grade 1 - Chronic inflammatory infiltrate
1.0 No increase | 1.1 Mild increase | 1.2 Moderate increase |1.3 Marked increase
Grade 2- Lamina propria neutrophils and eosinophils
2A Eosinophils 2A.0 No increase | 2A.1 Mild increase | 2A.2 Moderate increase | 2A.3 Marked increase
*2B Neutrophils 2*B.0 None | 2B.1 Mild but unequivocal increase | 2B.2 Moderate increase | 2B.3 Marked increase
Grade 3 - Neutrophils in epithelium
3.0 None | 3.1 < 5% crypts involved | 3.2 < 50% crypts involved | 3.3 ≥ 50% crypts involved
Grade 4 - Crypt destruction
4.0 None 4.1 Probable—local excess of neutrophils in part of crypt| 4.2 Probable—marked attenuation| 4.3 Unequivocal crypt destruction
Grade 5 - Erosion or ulceration
5.0 No erosion, ulceration, or granulation tissue | 5.1 Recovering epithelium adjacent inflammation | 5.2 Probable erosion/focally stripped | 5.3 Unequivocal erosion | 5.4 Ulcer or granulation tissue

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

	Surface epithelium	Crypt epithelium	Crypt abscesses
1 O	X		
2 O		X	
3 O	X	X	
4 O			X
5 O	X		X
6 O		X	X
7 O	X	X	X

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[61]. 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[62].

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)[45, 63]. Surgery is often the treatment of last resort when there is no clinical response to other treatments, including intravenous CSs[63].

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, Mesasal or Asacol) and azo-compounds that are delivered in the colon by bacterial cleavage (sulfasalazine)[41]. 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[64, 65].

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 treated with suppository or enemas[40, 41]. An ongoing discussion concerns whether 5-ASA should be given in low or high dose, with some reports claiming that high dose or low dose regimes are equally effective as short-term treatment of active UC in children[66, 67].

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[68]. Through a mechanism termed transactivation, a cytoplasmic intracellular receptor for glucocorticosteroids affect a glucocorticoid-responsive element in the DNA, which induces the production of anti-inflammatory proteins[69]. Transactivation is also responsible to the CS side-effects by an induction of gluconeogeneic enzymes[68]. The receptor also binds to glucocorticosteroids-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-κβ[70], and further, the receptor complex competitively inhibits co-activators in the nucleus followed by reduced expression of cytokines, like TNF-α and IFN-γ[68].

CSs are recommended for children with moderate to severe UC with systemic symptoms[41] 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%[67, 71, 72]. Nevertheless, the correlation between clinical remission and endoscopic remission after cortisone treatment is low[73]. In children with CD, EEN is the first-line treatment, but CS therapy may be a viable alternative[40].

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[74]. If CSs must be used, the treatment should be tapered as soon as possible because of the risk of these side effects[41, 72]. 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[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)[40]. 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[40, 76-79]. 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[40]. 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[80, 81, 82]. 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[75]. 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-Mercantopurine

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[83].

Small, retrospective studies[84, 85] have shown that Azathioprines (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[41]. 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[86]. 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

hepatosplenic T-cells lymphoma[87-89]. Thiopurins is considered to reduce the risk of development of antibodies to Infliximab (ATI) and is therefore often used as a concurrent treatment to IFX during the first six month of Infliximab treatment (when the risk is considered high), but even here the reports are contradictive[90-92].

1.7.5 Anti-TNF-alpha treatment (infliximab and adalimumab)

Infliximab (IFX, Remicade®) is a chimeric monoclonal IgG_1 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 [90, 93]. 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[40]. One study showed that IFX maintenance therapy was effective, being associated with prolonged CS withdrawal over a 3-year period in children with CD[94]. 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)[95]. 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[96]. Other malignancies or Hemophagocytic Lymphohistiocytosis (HLH) are not associated with anti-TNF treatment in children according to a new study from 2017[97].

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)[90, 98-100]. If the s-IFX trough level is low, the anti-TNF dosage and/or IFX infusion intervals should be adjusted[40]. Loss of response can occur because of antibodies against IFX and because the inflammatory burden is very extensive[90, 101]. ATI can also produce acute infusion reactions and delayed-type hypersensitivity reactions[40]. Most studies on IFX trough

levels and ATI have been performed in adult IBD patients, and the pediatric studies on IFX are quite few in small study populations.

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 acetate cellulose beads that attract and remove the Fc-ɣ receptors of activated leukocytes (granulocytes and monocytes) with a granulocyte adsorption ability over 2.18 x 10^9 cells during one GMA session[102-104]. 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[105].

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[106, 107]. Some studies suggest that more intense treatment (two to three sessions a week instead of the conventional once a week) is more effective in inducing remission[108, 109].

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[110]. Only a few previous reports are published considered GMA in children.

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[111]. 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.[112]

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[111]. 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[113]. 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[114].

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.[115]

An imbalance in the composition of the intestinal bacterial, 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[116]. 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[117]. 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[118]. 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[119].

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 homoeostasis 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[120]. 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: xenobic metabolism (metabolism of toxins and drugs), mucosal barrier strengthening, nutrient absorption, intestinal maturation and angiogenesis[121]. 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)[120]. 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[122] . The Paneth cells secret the antimicrobial protein α-defensin (small cysteine-rich cationic proteins). White

blood cells and mucosal epithelial cells in entire GI tract secret β-defensins, another antimicrobialprotein. The defensins exert their effect through lysate bacterial membranes with their amphipathic properties[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[120]. TLRs trigger both innate and adaptive responses to microbes initiating an intracellular signaling process that results in a cytokine cascade leading to inflammation[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[126,127]. Conversely, TLRs have been shown to contribute to intestinal homeostasis when not triggered by pathogens[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 NKCs. The microfold cells (M cells) in the villi 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[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[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[132]. This process represents an important aspect of how the immune homoeostasis of the healthy GI tracts is achieved.

1.9.5 Cytokines

Cytokines refers 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[133, 134]. The first cytokine was identified 1957 (interferon type I), and now decades later a broad spectra of cytokines like interferons (IFN), interleukins (IL), chemokines, mesenchymal growth factors (MGF), the tumor necrosis factor family (TNF) and adipokines are described[135]. 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[136]. 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[137-139]. An overview over the complex cytokine landscape in table 5.

1.9.6 Chemokines and chemokine receptors

Chemokines (*chemotactic 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)[136]. To date, 23 CCRs that may exert chemotaxis have been identified, together with some 50 chemokine ligands[140]. 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[141].

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

CYTOKINE	MAIN SOURCE	TARGET CELL	FUNCTION
IL-1α, IL-1β, IL-1ra, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37	MΘ, B-cells, DC	B-cells, NK, T-cells	Pyrogenic,pro-infl, proliferation, differentiation, BM cell profileration
IL-2, IL-21	T-cells	activated T -and B-cells, NK	Proliferation and activation
IL-3	T-cells, NK	stem cells	Hematopoetic precursor, proliferation and differentiation
IL-4	Th cells	B-cells, T-cells, MΘ	Proliferation of B-cells andTcyt, enhances MHC class.II expression, stimulates IgG and IgE production
IL-5	Th	Eosinophils, B-cells	Proliferation and maturation, stimulates IgA and IgM production
IL-6, IL-11, IL-31, CNTF, CT-1, LIF, OPN, OSM	Th, MΘ, fibroblasts	Activated B-cells, plasma cells	Differentiatin into plasma cells, IgG production
IL-7	BM stromal cells, epithelial cells	Stem cells	B- and T cell growth factor
IL-8	MΘ	Neutrophils	Chemotaxis, pro-inflammatory
IL-9	T-cell	T-cell	Growth and proliferation
IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29	T-cell	B-cells, MΘ	Inhibits cytokine production and mononuclear cell function, anti-inflammatory
IL-12, IL-23, IL-27, IL-35	T-cells	NK-cells	Activates NK-cells
TNF-α	MΘ,monocytes	MΘ, tumor cells	Phagocyte cell activation, endotoxic shock, tumor cytotoxicity, cachexia
TNF-β	T-cells	Phagocytes, tumor cells	Chemotactic, phagocytosis, oncostatic, induces other cytokines
IFN-α	Leukocytes	Various	Anti-viral
IFN-β	Fibroblasts	Various	Anti-viral, anti-proliferative
IFN-γ	T-cells	Various	Anti-viral, MΘ activation, increases funtion of neutrofiles and monocytes
G-CSF	Fibroblasts, endothelium	Stemcells in BM	Granulocyte production
GM-CSF	T-cells, MΘ, fibroblasts	Stemcells	Granulocyte, monocyte, eosinophil production
M-CSF	Fibroblasts, endothelium	Stemcells	monocyte production and activation
ERYTROPOETIN	Endothelium	Stemcells	Red blood cell production
TGF-β	T-cells, B-cells	Activated T-cells and B-cells	Inhibit T and B cell proliferation, inhibit hematopoiesis, promote wound healing

MΘ=macrophage, Th= T helper cell, NK= natural killer cell, DC= dendritic cell, BM= bone marrow

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 barrier[37]. T-cells mediated disruption of the tight junction and dysfunction of the enteric neuron are the supposed mechanism for this permeability[142]. 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 CD[143].

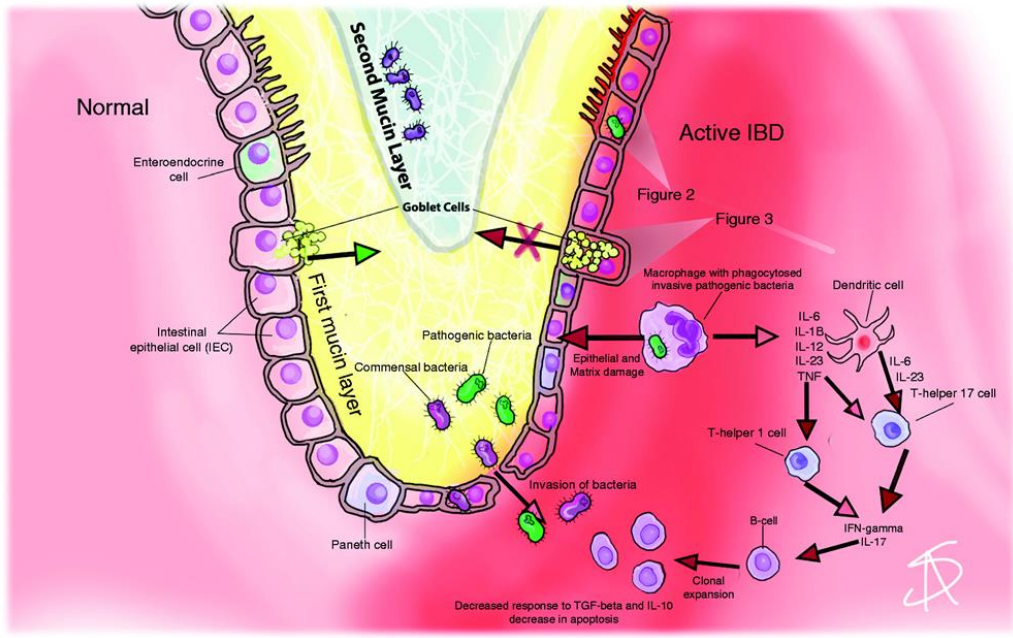
NOD2 is an intracellular PRR encoded by the NOD2 gene (chromosome 16). It recognizes peptidoglycans in the cell wall of bacteria and stimulates immune responses. Reduced NOD2 function may be followed by the inhibition of TLR stimulation, leading to extremeTh-1 response through different inflammatory 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[120]. 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[120].

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) [148]. CD is considered driven by a Th1 immune response, whereas UC is known as a Th2-mediated disease[149]. 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[24].



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 indicies 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.

3 CHILDREN AND METHODS

An overview of the study design, methods and participants are presented in Table 5.

Paper	Design	Study population at inclusion	Number of participants	Aim	Method	Statistical method
I	Cohort	Children with IBD on maintenance IFX treatment	45	To study s-IFX trough levels, ATI and associations with clinical activity.	In-house developed ELISA, PUCAI/PCDAI, blood chemistry, f-calprotectin	Pearson with cluster-robust standard error. Student T-test.
II	Cohort (pilot)	Children with new onset IBD colitis	13	To study the clinical effect of GMA and mesalazine as induction of remission	PUCAI, Geboes score, endoscopic Mayo, blood chemistry, f-calprotectin	Paired sample t-test, McNemar test
III	Cohort (pilot)	Children with new onset UC, CD and non-IBD controls	45	To distinguish CD from UC by expression of chemokine receptor pattern on blood leukocytes	Flow cytometry, PUCAI/PCDAI, blood chemistry, F-calprotectin	Univariate logistic regression, multivariate logistic regression
IV	Cohort	Children with new onset IBD and non-IBD controls	19	To study the clinical outcome of EEN and changes in the colonic mucosal cytokine profiles	PCDAI, Geboes score, SES-CD, blood chemistry, f-calprotectin. Real time PCR	Paired sample t-tests, Wilcoxon paired t-test, Mann-Whitney U test.
V	Cohort	Children with new onset IBD colitis and non-IBD controls	13 (7 IBD patients from study II)	To study changes in the colonic mucosal cytokine profiles at onset and after GMA	PUCAI, Geboes score endoscopic Mayo, blood chemistry, f-calprotectin. Real time PCR	Paired sample t-tests, Wilcoxon paired t-test, Mann-Whitney U test.

Table 5. Overview of study design, method and study population.

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, Västerås, 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.

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[44]. 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[44] 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 guildelines[44]. 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

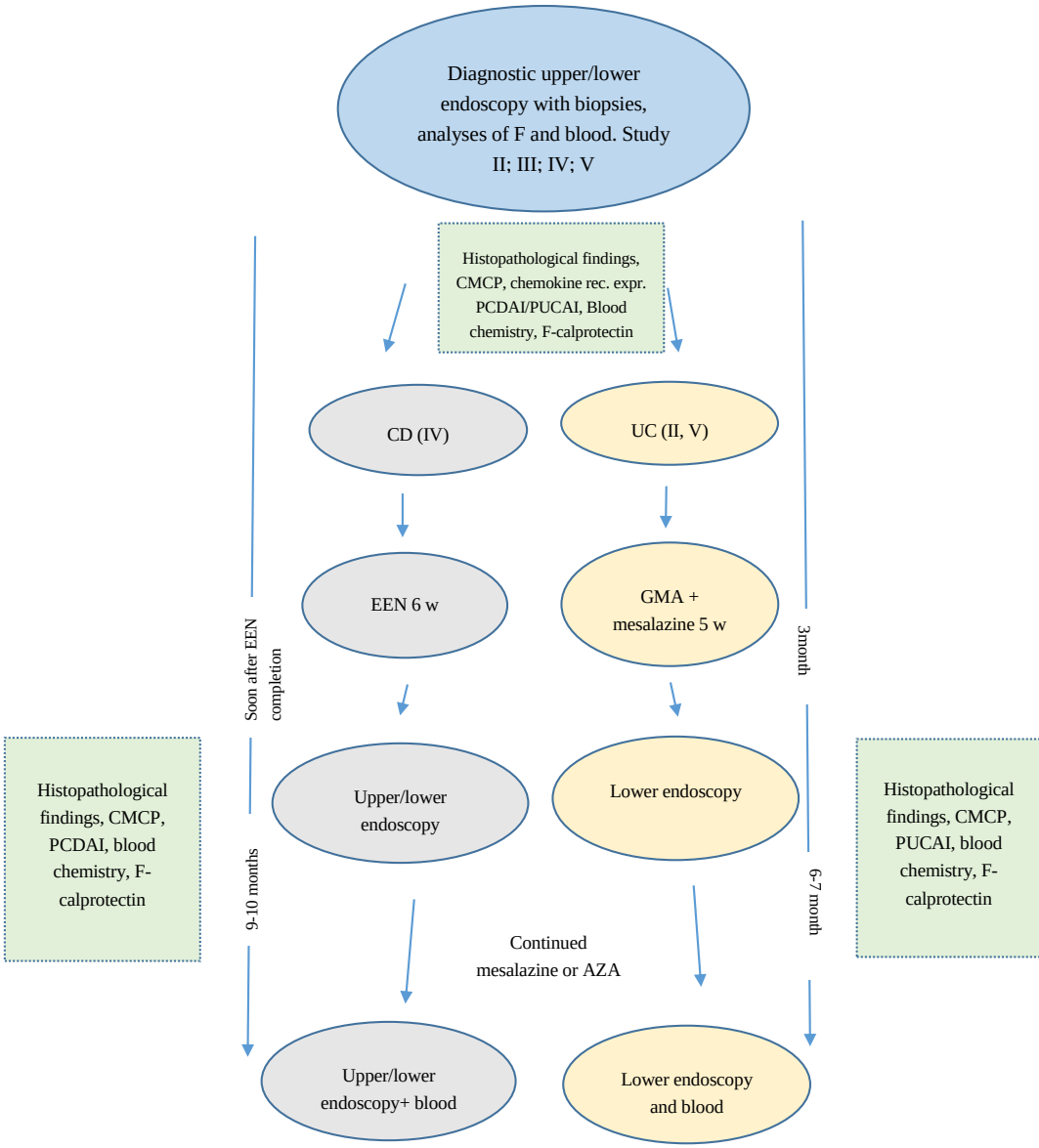
3.2.1 Paper I

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[45], CRP <5[150] and ESR <10[151]; UC patients were considered in remission if the PUCAI was <10[47], CRP <5 [152] <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[153]. 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.

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)



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, PCDAI 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.

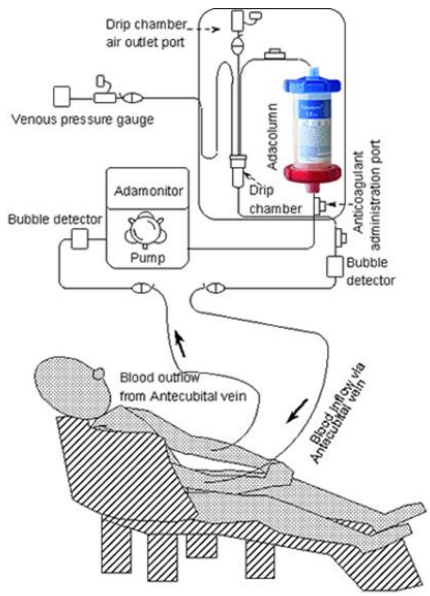
3.2.5 Paper V

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.

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)



An outline of the extracorporeal circulation through the Adacolumn.

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-23α, IL-36γ, 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 ºC 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: $\Delta Ct = Ct$ (gene of interest) $- Ct$ (ABL1), $\Delta\Delta Ct = \Delta Ct$ sample $-$ average ΔCt control group and fold difference $= 2^{-\Delta\Delta Ct}$. 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.

P**aper II:** The Shapiro Wilks test was used for normality testing in all parameters and comparisons between ESR, CRP, albumin, Hb, F-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 (CD3+), 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 I**, 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).

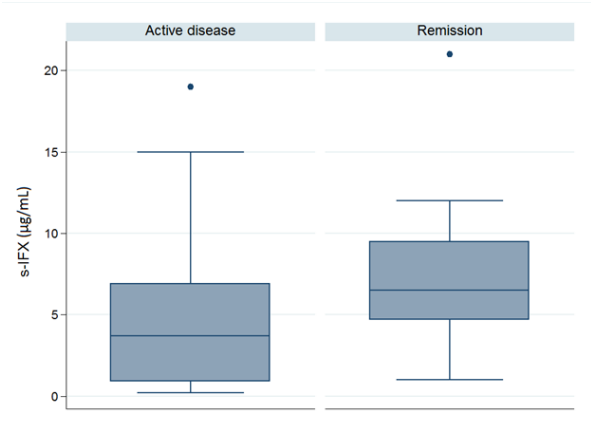


Figure 5. The mean trough s-IFX level was significantly higher in the samples taken during remission (7.2 µg/mL) as compared with s-IFX in active disease (4.5 µg/mL) ($p<0.05$).

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

prematurely retired from the study owing to non-response. At onset, extensive or pancolitis was found in all patients. No serious adverse events occurred during the treatment period.

Throughout the treatment, none of the patients showed a worsening of symptoms. In the time between complete ADA treatment and CC, three patients had a flare (after 4, 15 and 62 days) and were treated with prednisolone (1.5 mg/kg, 0.5 mg/kg and 0.3 mg/kg, respectively) for 4-6 weeks. One of the flaring patients also suffered from concurrent pancreatitis.

Disease activity: A cross-table analysis (McNemar test) with two subgroups at diagnosis revealed that patients with a PUCAI <35 (none to mild disease) and PUCAI ≥35 (moderate to severe disease) demonstrated a significant decrease in PUCAI at the time of CC (p=0.004). (Table 6). Of the three prednisolone treated patients, one was in clinical remission and two were in active disease. (Table 6).

Table 6. Disease activity: PUCAI scoring at the time of diagnosis, the 10th ADA and the CC.

	PUCAI at diagnosis N=13	PUCAI at 10th ADA N=12	PUCAI at control colonoscopy N=12
Remission <10 p	0	6	8
Mild 10-34 p	1	4	2
Moderate 35-64 p	10	2	2
Severe ≥65- 85	2	0	0

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 10th 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 azathioprine 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 7. Laboratory values, weight and height at inclusion and at control colonoscopy

Value	At inclusion Median (IQR) range	After EEN treatment Median (IQR) range	P-value
Weight (kg)	37.9 (31.5-52.6) 27.5-59.7	40.5 (32.8-55.4) 29.8-59.1	0.003
Height (cm)	152 (148.5-165.6) 139-172.5	152.8 (147.7-166.0) 139.5-172.5	0.027
ESR (mm/h)	28 (17.3-27.5) 8-53	9 (8-11) 2-21	0.005
CRP (mg/L)	14 (2-49.3) 1-86	2.5 (1-8.3) 1-20	0.016
Albumin (g/L)	33 (25-36) 24-41	36.3 (33.5-40.5) 31-44	0.003
Hemoglobin (g/L)	113 (101-119.5) 83-142	129 (121-135) 116-144	O.001
F-calpro (mg/kg)	2640 (1191-3169) 580-6360	620 (198-1556) 5-2256	0.033

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

Geboes score with an addition for CD characteristics. The Geboes score showed improvements in 10 patients, worsening in 1 and no inflammation in 1.

4.5 COLONIC MUCOSAL CYTOKINE PROFILES AND CLINCAL 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,

IQR 0-3, range 0-6) as well as ESR (29-9.7, p=0.048), F-calprotectin (3825-872, p=0.032) and a significant raise of albumin (35-40.6, p=0.015).

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.

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[99, 100, 154]. 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[92, 154, 155]. 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[156].

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[101]. 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[165]. 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[91]. 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.

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[166, 167]. 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[168]. 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[169].

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[107]. The authors discuss the importance of giving GMA early in the disease course. Previous studies (20 to 40 patients included) have shown that steroid naive (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 [106, 110, 170-172].

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. Ruuska et al, for instance, showed significantly reduced steroid dosage in 37 steroid-dependent children after five to nine GMA sessions[174]. In addition, some other, small studies (four to nine patients) have reported encouraging results[175, 176].

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 (ileal 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[28].

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-23α, IL-1β, IL-36γ, IL-10 and TGFβ1 and non-significant decreases in IL-4, IL-5 and IL-6. We also found significant higher levels of IL-12β and IL-23α as well as non-significant higher values of IL-22 and IL-36γ 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 CSs in a standard care setting. Three children also received CSs 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[178,133]. 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.

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 CSs[40, 41]. 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 entities[179]. Even more troubling is that a delayed diagnosis may be followed by a lag in pubertal growth spurt and impaired adult height[180]. 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 controls[181]. 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)[182]. 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 guidelines[44], with a

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-naïve children.

5.4 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[40], 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[79, 183, 184]. Furthermore, one study showed nearly equal effectiveness between anti-TNF and EEN in clinical outcomes (PCDAI) and mucosal healing as estimated by fecal calprotectin[185]. 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[186]. It has previously been shown that pediatric gastroenterologists in North America found their concern of patient adherence as the main disadvantage with EEN[187]. 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[188]. 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

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[76]. 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[189]. If the new nomenclature according to Cleynen et al is implemented[28], 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-12β and IL-23α were observed in the IBD patients. These cytokines play an important role in the pathogenesis of CD[190]. 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[191]. 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)[192]. 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[28, 193]. 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[194].

> "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 investigate 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

Crohns sjukdom (CD) och ulcerös kolit (UC) utgör tillsammans gruppen kronisk inflammatorisk tarmsjukdom, som ofta benämns IBD, efter engelskans ”inflammatory bowel disease”. I Sverige är ca 0,65 % av befolkningen drabbad, och ca 10-20 % av de drabbade insjuknar under barndomen. 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 i IBD, men en kombination av arv, immunologiska faktorer och miljöfaktorer tros samverka.

Symtomen är liknande för UC och CD med buksmärtor, (blodiga) diarréer, illamående, trötthet, dålig ork, uppblåst mage, viktnedgång och för barn ibland dålig tillväxt. Man kan även vid CD få fistelgångar mellan tarm och hud, anala bölder eller trånga partier i tarmen. Andra organ i buken som lever och gallvägar kan påverkas liksom symtom utanför magtarmkanalen kan uppstå som hudutslag, ledvärk, ögonbesvär och munsår.

Sjukdomsförloppet kännetecknas av att symtomen kommer och går, och när man får mycket symtom kallas de för att man får skov. Barn har oftare ett aggressivare förlopp än vuxna, och eftersom barnaåren är en viktig och kort tid i livet när tillväxt, skola, sociala kontakter och självbild skall utvecklas, bör skov undvikas, varför de allra flesta barn har en pågående behandling för att förhindra nya inflammationsutbrott, s.k. underhållsbehandling.

Om barnet trots allt får ett skov, vilket är ganska vanligt trots underhållsbehandling, behandlar man med en s.k. remissions behandling för att dämpa inflammationsutbrottet, och inte sällan består denna behandling av höga doser kortison, vilket kan resultera i allvarliga biverkningar på både kort och lång sikt som t.ex. viktuppgång, svår acne, hudbristningar, psykisk oro, magont, sömnlöshet, högt blodtryck och benskörhet.

Alla barn som misstänks ha IBD lämnar blodprover och genomgår en gastroskopi och koloskopi där små vävnadsprover tas på många ställen i magsäcken och tarmen och undersöks i mikroskop av en patolog som kan finna särskilda karaktäristika tillhörande de olika sjukdomarna.

I **studie 1** undersökte vi hur ett exklusivt, dyrt läkemedel; infliximab (IFX) (Remicade®, Inflectra®, Humira®) fungerar hos barn som behandlas med IFX som underhållsbehandling. Det är ett biologiskt aktivt läkemedel som består av antikroppar mot ett inflammationsämne (en cytokin) som heter TNF-α, vilket bildas vid inflammation. IFX hämmar TNF-molekylen så den inte kan skapa mer inflammation. Att IFX är biologiskt aktivt innebär tyvärr att kroppen kan reagera mot läkemedlet och uppfatta det som en fiende som skall bekämpas. Detta gör att det är svårt att förutse hur mycket läkemedel en enskild patient behöver, samt att man kan få en

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 IBD-patienterna har kvar i blodet när de kom för att få sin nästa infusion (dalvärde). Vi kontrollerade sänka och CRP, och de fick fylla i formulär hur de mådde (klinisk scoring) Vi fann att en tredjedel av patienterna hade aktiv inflammation och inte mådde bra trots behandlingen, och att de som utvecklat antikroppar mot IFX inte 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 monocyt 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 inflammations celler som cirkulerar i blodbanan som annars skulle vandra ner till tjocktarmen och skapa inflammation. Tolv barn och ungdomar över 12 år, fick 10 behandlingar under 5 veckor samt en mild tilläggsbehandling med meslazin (studie II). Barnen genomgick en kontroll koloskopi ca tre månader efter den tionde 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 tarmslemhinna, och att 8 av 12 barn tyckte de kände sig helt bra (klinisk remission) samt tydliga nedgångar i inflammationsprover i blodet. Vid undersökning av cytokinerna i tarmslemhinnan 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 spekulerar i att nedgångarna i tarmslemhinnan förklarar det goda behandlingssvaret.

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 algoritm utifrån chemokin-receptor mönstret på fyra olika vita blodkroppar utarbetades varpå vi kunde särskilja UC från CD. Detta är en pilotstudie för att utveckla en prognostisk markör då vi i ca 10 % av fallen har svårt att korrekt diagnostisera UC från CD, viket gör att vissa patienter kan få en felaktig eller sämre behandling än om de haft rätt diagnos från början.

I **studie IV** behandlade vi 12 barn nydiagnostiserade med CD och behandlade dem med flytande kostbehandling, s.k. exklusiv enteral nutritionsbehandling (EEN) vilket innebär att barnet enbart 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 biverkningsfri och läker tarmen bättre än kortison. Vi ville undersöka hur våra patienter svarade på behandlingen, samt

hur cytokinerna såg ut i tarmslemhinnan 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 tarmslemhinnan. 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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