Studies of experimental biomarkers during glucocorticoid and anti-tumor necrosis factor-alpha therapy in inflammatory bowel disease and juvenile idiopathic arthritis

Hanne Rintamäki

ACADEMIC DISSERTATION

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To Kaija-Leena
Abstract

Anti-inflammatory medicines, such as glucocorticoids (GCs) and anti-tumor necrosis factor-α (anti-TNF-α) agents are used for treatment of inflammatory bowel diseases (IBD), Crohn’s disease (CD) and ulcerative colitis (UC), and also in juvenile idiopathic arthritis (JIA). GCs are used for treatment of moderate to severe adult and pediatric IBD. The use of GC is restricted by inadequate response or development of steroid dependency. In addition, GCs can induce adverse effects such as suppression of the hypothalamic-pituitary-adrenal axis. In JIA, intra-articular corticosteroid therapy (IACS) is used for treatment of oligoarticular disease or flare-ups of independent joints. Anti-TNF-α agents are used for treatment of GC refractory IBD. The anti-TNF-α agent infliximab (IFX) is indicated for use in pediatric IBD. Anti-TNF-α agents can also have severe adverse effects including infections and malignancies. A laboratory test in clinical use to predict those patients who would respond to GC or anti-TNF-α therapy would be important to guide the therapeutic use of these agents. In this thesis, the attenuation of inflammation during GC and anti-TNF-α therapies was evaluated by using experimental biomarkers in aim to find new tools to optimize GC and anti-TNF-α therapy in pediatric and adult IBD patients, and in JIA patients receiving IACS.

The serum samples were prospectively collected from IBD patients before the start of oral GC (I, IV) or anti-TNF-α therapy (III, IV), and from the pediatric controls (IV). The second sample was collected from pediatric IBD patients two to four weeks after the start of GC therapy (I, IV), and at week two after the first IFX infusion (IV). In all, 57 pediatric IBD patients were recruited from the Children’s Hospital, University of Helsinki. Twenty-seven children had oral prednisolone, and one had budesonide (I, IV). Sixteen pediatric IBD patients received IFX (IV). Fifteen adult CD patients from the Clinic of Gastroenterology, Helsinki University Central Hospital had therapy induction with either IFX or adalimumab (III). Thirteen pediatric IBD patients with quiescent disease and 19 other pediatric patients served as controls (IV). In addition, 21 JIA patients were prospectively recruited from Heinola Rheumatism Foundation Hospital, and had 2-7 joints injected at one time with methylprednisolone (MP) or MP in combination with triamcinolone hexacetonide (II). The JIA patients provided serum samples on the morning before the IACS, seven and 24 hours thereafter, and at two months.

A new biological assay for monitoring the patient serum immune-activation potency was developed (I-III). The assay is based on analysis of the effect of patient serum on donor derived allogenic peripheral blood mononuclear cells, refered here as target cells. As read-outs, the markers of T-cell activation, interferon-gamma (IFNγ), interleukin-5 (IL-5), forkhead transcription factor 3 (FOXP3) and glucocorticoid-induced tumor necrosis factor receptor (GITR), were analyzed in the culture supernatants with enzyme-linked immunosorbent assay (ELISA) or in the target cells with polymerase chain reaction. In addition, serum glucocorticoid bioactivity (GBA) was measured from the JIA patients (II). In the work IV, matrix metalloproteinases (MMP-7-9), their tissue inhibitors (TIMP-1-2), alpha-2-macroglobulin (α2M), human neutrophil elastase (HNE) and myeloperoxidase (MPO) were measured from the serum with ELISA.
In the first study, the serum immune-activation potency was studied in pediatric IBD patients at the start of oral GC therapy and two to four weeks thereafter (I). In comparison with pre-treatment serum, patient serum after the treatment reduced target cell IFNγ secretion (p=0.017 for naïve cells and p=0.006 for activated cells) and GITR expression (p=0.033 and p=0.005 respectively). The decrease on serum induced FOXP3 expression was seen on naïve target cells (p=0.05). These changes did not correlate with weight-related GC dose, thus the observed changes represented the sum effect of individual inflammatory activity in patient serum, rather than GC concentration.

In the second study with JIA patients (II), IACS increased serum GBA (p=0.001) and decreased cortisol (p=0.002) within 24 hours of the injection. In the target cell assay, the patient serum induced IL-5 secretion showed a trend of decrease (p=0.085). These findings indicate a leakage of biologically active GC from the joint.

In the third study, the serum immune-activation potency was studied in adult CD patients at the start of anti-TNF-α therapy and its association with endoscopic disease activity (CDEIS) within three months (III). Before the start of the therapy, the high serum induced FOXP3 and GITR expression on activated target cells were associated with low CDEIS (r=-0.621, p=0.013 for FOXP3 and r=-0.625, p=0.013 for GITR) and to low erythrocyte sedimentation rate (r=-0.548, p=0.034 for FOXP3). Interestingly, the low pre-treatment FOXP3 and GITR expression on target cells were associated with a pronounced decrease of CDEIS during the therapy (r=0.600, p=0.018 for FOXP3 and r=0.589, p=0.021 for GITR). Restoring the number and function of regulatory T-cells (Tregs) is one central therapeutic effect of anti-TNF-α agents. It is possible that there is a group of IBD patients who do not benefit from additional enhancement of Tregs induced by anti-TNF-α agents.

In the final study, serum MMPs, TIMPs, α2M, HNE and MPO were measured from pediatric IBD patients during GC and anti-TNF-α therapies (IV). The serum level of MMP-7, MMP-8, MMP-9, α2M, HNE, MPO in all IBD patients and TIMP-1 in the GC treatment group was higher than in the controls (all p≤0.022). During the GC therapy, the attenuation of inflammation was seen as a decrease of MMP-7 and TIMP-1 (both p≤0.001). During the anti-TNF-α therapy, α2M and HNE increased (both p≤0.026). As the α2M is the major plasma inhibitor of MMPs, its increase was suggested as an additional mechanism mediating anti-TNF-α agent’s therapeutic effect.

Taken together, studying the patient serum immune-activation potency and measurement of serum MMPs, TIMPs, α2M and HNE seemed promising approaches to explore the anti-inflammatory effects of GC and anti-TNF-α therapies in IBD and JIA. It should be noted that the number of patients was relatively small, thus further studies are needed to evaluate the clinical usefulness of the proposed means to monitor the treatment response during GC and anti-TNF-α therapies.
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Acknowledgements

References
List of original publications

This thesis is based on the following publications that are referred to in the text by their roman numerals:


IV  Mäkitalo L, Rintamäki H, Tervahartiala T, Sorsa T, Kolho KL. Serum MMPs 7-9 and their inhibitors during glucocorticoid and anti-TNF-α therapy in pediatric inflammatory bowel disease. In press.

These articles were reprinted with the copyright holder’s permission. Some unpublished data are also presented.
Abbreviations

\( \alpha_2 \text{M} \quad \alpha_2 \)-macroglobulin
Ab antibody
ACTH adrenocorticotropin hormone
ANA antinuclear antibodies
ANCA antineutrophil cytoplasmic antibodies
Anti-OmpC anti-outer membrane porin C IgA antibody
Anti-TNF-\( \alpha \) anti-tumor necrosis factor-alpha
APC antigen presenting cell
ASCA anti-\textit{Saccharomyces cerevisiae} antibodies
BSA bovine serum albumin
CCP cyclic citrullinated peptide
CD Crohn’s disease
CD (4 etc.) cluster of differentiation
CDAI Crohn’s disease activity index
CDEIS Crohn’s Disease Endoscopic Index of Severity
CHAQ Child Health Assessment Questionnaire
CRP C-reactive protein
Ct threshold cycle
CTLA cytotoxic T lymphocyte associated antigen
DC dendritic cell
DEX dexamethasone
DMARD disease modifying antirheumatic drug
ECM extracellular matrix
ELISA enzyme-linked immunoabsorbent assay
ESR erythrocyte sedimentation rate
F-calprotectin fecal calprotectin
FOXP3 forkhead transcription factor 3 / forkhead box P3
GBA glucocorticoid bioactivity
GC glucocorticoid
GI gastrointestinal
GITR glucocorticoid-induced tumor necrosis factor receptor
GM-CSF granulocyte-macrophage-colony stimulating factor
GR glucocorticoid receptor
GRE glucocorticoid response element
HAT histone acetyltransferase
HDAC2 histone deacetylase
HLA human leukocyte antigen
HNE human neutrophil elastase
HPA hypothalamic-pituitary-adrenal
HsCRP high sensitive C-reactive protein
IACS intra-articular corticosteroids
IBD inflammatory bowel disease
IC indeterminate colitis
IFNγ interferon gamma
IFX infliximab
Ig immunoglobulin
IKKβ inhibitor of nuclear factor kappa-beta kinase
IL interleukin
IPEX immune dysregulation, polyendocrinopathy autoimmune enteropathy X-linked syndrome
JIA juvenile idiopathic arthritis
MHC major histocompatibility complex
MMP matrix metalloproteinase
MP methyprednisolone
MPO myeloperoxidase
MR mineralocorticoid receptor
(m)RNA (messenger) ribonucleic acid
MT metallothionein
NFκB nuclear factor kappa-beta
NOD (2) nucleotide-binding-oligomerisation-domain (2)
NO(S) nitric oxide (synthase)
PBMC peripheral blood mononuclear cell
PBS phosphate buffered saline
PCR polymerase chain reaction
PGA Physician’s global asessment
PHA phytohemagglutinin
RA rheumatoid arthritis
RF rheumatoid factor
RORC2 retinoic acid receptor-related orphan receptor C2
RT-PCR reverse transcriptase polymerase chain reaction
SF synovial fluid
SFMC synovial fluid mononuclear cells
STAT signal transducer and activator of transcription
SuPAR soluble urokinase plasminogen activator receptor
TA triamcinolone acetonide
TCR T-cell receptor
TGF-β transforming growth factor-beta
Th T-helper
THA triamcinolone hexacetonide
TIMP tissue inhibitor of matrix metalloproteinases
TLR toll-like receptor
TNF tumor necrosis factor
Treg regulatory T-cell
UC ulcerative colitis
Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) are termed inflammatory bowel diseases (IBD). The classical symptoms of IBD are diarrhea, stomach ache and rectal bleeding. Intermittent fever can be related to Crohn’s disease. Extra-intestinal manifestations can also exist, joint symptoms being the most common. In addition, IBD can affect growth, fertility and mood. Juvenile idiopathic arthritis (JIA) is classified in seven subtypes, and the clinical picture varies from inflammation of a single joint to the systemic disease.

The exact etiology of IBD and JIA are unknown, but they both seem to have a multifactorial background. In IBD, it is suggested that genetic vulnerability together with environmental triggers lead to altered immune response against normally tolerated commensal flora. Disturbance of tolerance plays a role in immunopathogenesis of both diseases, and inflammatory activity of the patient’s white cells contributes to tissue damage. Recently, the balance between T-helper (Th) cells, especially Th17 cells and regulatory T-cells (Tregs), has received much attention in the pathogenesis of both IBD and JIA.

Anti-inflammatory medicines, such as glucocorticoids (GCs) and anti-tumor necrosis factor-alpha (anti-TNF-α) agents are used to induce and maintain remission in both IBD and JIA. The mechanisms of action of GCs are not fully understood despite the long history of GCs in the treatment of inflammatory diseases. The GC therapeutic effect seems to arise from the inhibition of inflammatory gene reading. Restoring the balance between Th-cells and Tregs has been shown as one therapeutic effect of anti-TNF-α agents. Up to 30% of adult IBD patients have been reported as treatment failures during systemic GC therapy, and a similar number of adult patients have become steroid dependent. Among pediatric IBD patients, the steroid dependency can be even more common. During anti-TNF-α therapy, approximately 60% of adult IBD patients have been reported to be short-term responders, and around 40% of the patients were able to maintain remission for one year. In addition, both GC and anti-TNF-α therapies can have severe adverse effects. Side-effects have been described even after local delivery such as intra-articular GC injections in JIA.

Several approaches have been tried to find early predictors for therapeutic response to GC therapy. Of the laboratory markers, glucocorticoid receptor (GR) isotypes and GR polymorphisms has been shown to be the most promising predictor in IBD. It has also been shown that parameters of lymphocyte function can reflect individual steroid sensitivity. There are very few studies exploring markers to predict anti-TNF-α therapy effects. High pre-treatment CRP associated with good therapeutic response to anti-TNF-α agents in adult IBD. In another study with adult CD patients, the high mucosal expression of pro-inflammatory transcription factor nuclear factor kappa-beta (NFκB) preceeded therapy relapse. There is no laboratory marker in clinical use to foresee those patients who would benefit from GC or anti-TNF-α therapy. Due to a fear of side-effects, this would be especially useful in pediatric patients.

In this thesis, a new biological assay was developed with the aim to find innovative tools for predicting therapeutic response for GC and anti-TNF-α therapy. This assay is based on detection of the treatment effect on the patient’s serum on donor derived
allogenic white cells that are referred as target cells. Patient serum immune-activation potency is assessed by measuring a panel of Treg (forkhead transcription factor 3, FOXP3 and glucocorticoid-induced tumor necrosis factor receptor, GITR) and Th-cell (interferon gamma, IFNγ, interleukin-5 and IL-17) markers in target cells or culture supernatants of the target cells. Other experimental serum markers, including glucocorticoid bioactivity (GBA), matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), alpha-2-macroglobulin (α2M), human neutrophil elastase (HNE) and myeloperoxidase (MPO) were also studied with the aim to find new tools to optimize GC and anti-TNF-α therapy in IBD and JIA.
1 Inflammatory bowel diseases

1.1 Classification and clinical picture

Inflammatory bowel diseases (IBD) that are Crohn’s disease (CD) and ulcerative colitis (UC), are lifelong, systemic inflammatory diseases of the gastrointestinal (GI) tract. CD can affect the GI tract from the mouth to the anus. Histologically, the inflammation patches in CD are submucosal or transmural (IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition 2005, Baumgart DC 2007 b). The Vienna classification of CD divides the disease according to location into disease of the ileum, colon, ileocolon or upper GI-tract. CD is further subclassified according to phenotype into inflammatory, penetrating or stricturing disease (Gasche C 2000). In UC, the inflammation typically extends continuously from the rectum to the proximal colon (Baumgart DC 2007 b). Histologically, the inflammation in UC involves only mucosa (IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition 2005). UC can be further divided into left-sided colitis, where the inflammation is located distally to splenic flexure, and pancolitis (Kugathasan S 2003). Those patients with colonic histological findings of inflammation with architectural changes that cannot be defined as UC or CD and with no disease in the small bowel, are diagnosed as unclassified or indeterminated colitis (IC) (IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition 2005). In two pediatric studies, IC was diagnosed in 3.3-14% of the IBD patients (Hildebrand H 2003, Turunen P 2006), and in a study with an adult population in 7.8% (Rubin GP 2000). The peak incidence age for CD is 15-25 years and for UC 25-35 years (Vind I 2000, Jussila A 2012). Another peak incidence age is suggested to be in patients over 75 years (Vind I 2000). In pediatric studies, the mean age at diagnosis varies between 11 to 13.5 years (Hildebrand H 2003, Kugathasan S 2003, Turunen P 2006). In adults, UC predominates over CD in developing countries, but it seems that a pattern of IBD is evolving (Rubin GP 2000, Bernstein CN 2008). In pediatric studies, the prevalence of CD has risen above that of UC (Hildebrand H 2003, Kugathasan S 2003), although in Finland UC predominates over CD (Lehtinen P 2011).

In IBD, the remission – relapse cycles follow each other (Baumgart DC 2007 b, Shikhare G 2010). IBD can typically present with abdominal pain, diarrhea and rectal bleeding, but the clinical picture can vary depending on the localization of the inflammation (Table 1) (Baumgart DC 2007 b, Shikhare G 2010). Both adult and pediatric patients can have extra-intestinal manifestations such as arthritis, erythema nodosum, pyoderma gangrenosum, episcleritis, uveitis or pancreatitis (Table 1) (Shikhare G 2010). At the time of diagnosis, 13-88% of pediatric CD patients were reported to have impaired growth, meaning height
standard deviation scores ≤-1.96, or decrease in height velocity (Walters TD 2008). Delayed and prolonged puberty are also reported among IBD, and especially CD patients (Kirschner BS 2008).

**Table 1**

*Clinical symptoms and extra-intestinal manifestations in pediatric Crohn’s disease (CD) and ulcerative colitis (UC) (Kugathasan S 2003, Sawczenko A 2003, Gupta N 2008).*

<table>
<thead>
<tr>
<th>Symptom</th>
<th>CD (%)</th>
<th>UC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal bleeding</td>
<td>21-43</td>
<td>83-84</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>30-56</td>
<td>74-98</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>42-72</td>
<td>43-62</td>
</tr>
<tr>
<td>Weight loss</td>
<td>21-58</td>
<td>31-38</td>
</tr>
<tr>
<td>Fatigue</td>
<td>8-13</td>
<td>2</td>
</tr>
<tr>
<td>Fever</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>Aphthous ulcers</td>
<td>3-5</td>
<td>13</td>
</tr>
<tr>
<td>Anorexia</td>
<td>21-25</td>
<td>6</td>
</tr>
<tr>
<td>Lethargy</td>
<td>27</td>
<td>12</td>
</tr>
<tr>
<td>Nausea/Vomiting</td>
<td>6</td>
<td>0.6</td>
</tr>
<tr>
<td>Constipation/Soiling</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Growth failure/ Delayed puberty</td>
<td>4-8</td>
<td>-</td>
</tr>
<tr>
<td>Toxic megacolon</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>Perianal fistulae</td>
<td>4-8</td>
<td>-</td>
</tr>
<tr>
<td>Anal fissure or anal skin tags</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>Cholangitis/liver disease/abnormal liver enzymes</td>
<td>1-3</td>
<td>3</td>
</tr>
<tr>
<td>Arthritis or arthropathy</td>
<td>2-7</td>
<td>2-6</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Erythema nodusum/rash</td>
<td>1-2</td>
<td>0.6</td>
</tr>
<tr>
<td>Pyoderma gangrenosum</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

**1.2 Diagnosis**

IBD diagnosis is confirmed by endoscopy with biopsy, or imaging techniques such as wireless capsule endoscopy (Girardin M 2008, Bernstein CN 2010, Shikhare G 2010). Serological and fecal laboratory tests (Table 2) can provide information on the disease severity and activity, but the inflammatory parameters C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) can also be at normal levels (Bernstein CN 2010, Sidoroff M 2010, Turner D 2010 b). Fecal markers such as calprotectin (Røseth AG 1999) or lactoferrin (Kayazawa M 2002) can be used to monitor the inflammation of the gut (Fagerhol MK 2000, Bernstein CN 2010). In adult IBD patients, both fecal calprotectin and lactoferrin correlated with endoscopic score and colon histology (Sipponen T 2008 a, Langhorst J 2008). In pediatric IBD patients, fecal calprotectin correlated closely with macroscopical and histological inflammation (Bunn SK 2001, Kolho KL 2006).
Table 2  Laboratory tests for suspected inflammatory bowel disease. Modified from Strople J 2008.

<table>
<thead>
<tr>
<th>Test</th>
<th>Possible finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood count</td>
<td>Anemia, thrombocytosis, leukocytosis</td>
</tr>
<tr>
<td>Liver function test</td>
<td>Elevated transaminases, alkaline phosphatase or gamma-glutamyl transferase</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>Hypoalbuminea</td>
</tr>
<tr>
<td>ESR</td>
<td>Elevation</td>
</tr>
<tr>
<td>CRP</td>
<td>Elevation</td>
</tr>
<tr>
<td>Stool calprotectin</td>
<td>Elevation</td>
</tr>
<tr>
<td>Stool lactoferrin</td>
<td>Elevation</td>
</tr>
<tr>
<td>Serologic tests: ASCA, pANCA, anti-OmpC</td>
<td>May support the diagnosis</td>
</tr>
</tbody>
</table>

ESR= erythrocyte sedimentation rate, CRP= C-reactive protein, ASCA=anti-Saccharomyces cervisiae antibodies, pANCA=perinuclear antineutrophil cytoplasmic antibodies, anti-OmpC=anti-outer membrane porin C IgA antibody

1.3 Etiology

The etiology of IBD is considered to be multifactorial in that genetic susceptibility combined with environmental factors trigger a mucosal immune response against normally tolerated antigens like commensal flora (Baumgart DC 2007 a).

A positive family history is considered to be the largest independent risk of IBD (Baumgart DC 2007 a). In studies of twins, genetic concordance seems more abundant in CD than in UC (Tysk C 1988). In familial aggregation studies, 5-22.5% of the IBD patients are reported to have first degree relatives with IBD (Russell RK 2004). In pediatric studies, 11-18.5% of the IBD patients had first- or second-degree affected relatives (Kugathasan S 2003, Hildebrand H 2003, Turunen P 2009). Pediatric CD patients with early onset disease more frequently had first-degree relatives with IBD than older CD patients (42% versus 19%) (Weinstein TA 2003). The first identified genetic linkage was the association between CD and a mutation of the NOD2/CARD15 gene in chromosome 16 that codes for intra-cellular microbe sensing pattern recognition receptor, nucleotide-binding-oligomerisation-domain (NOD) 2 (Hugot JP 2001, Martinon F 2005). Today, the developing genome-wide association techniques have revealed over 60 IBD susceptible loci; potential candidate genes being for example \textit{IL23R}, \textit{IL27} and \textit{toll-like receptor-4 (TLR4)} (Henderson P 2011, Thompson AI 2011). Approximately half of IBD susceptible loci have been confirmed in both UC and CD (Thompson AI 2011), and 60% of adult loci have been replicated in pediatric studies (Henderson P 2011).
The IBD incidence rates are highest in Western countries (Baumgart DC 2007 a), and in most pediatric cohorts the incidence rates have continued to rise (Kugathasan S 2003, Hildebrand H 2003, Lehtinen P 2011). Increased numbers of IBD in Asian countries have been associated with socioeconomic changes in this area (Zheng JJ 2005). It is suggested that the slow genetic changes cannot account for the fast increase in IBD incidence which implies that environmental and lifestyle factors contribute to the IBD etiology (Bernstein CN 2008, Nunes T 2011). In epidemiological studies, several environmental risk factors such as host microbial environment, diet and smoking have been identified as modifying the development of IBD, but it should be emphasized that the studies are discrepant, and no risk factor with high impact has yet been identified (Table 3) (Bernstein CN 2008, Nunes T 2011).

### Table 3
*Role of environmental factors in development of ulcerative colitis (UC), Crohn’s disease (CD) and juvenile idiopathic arthritis (JIA) (Baumgart DC 2007 a, Bernstein CN 2008, Nunes T 2011, Ellis JA 2010).*

<table>
<thead>
<tr>
<th>Environmental risk factor</th>
<th>UC</th>
<th>CD</th>
<th>JIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longer duration of breast feeding</td>
<td>↓</td>
<td>↓</td>
<td>↓↑</td>
</tr>
<tr>
<td>Consumption of polyunsaturated fats</td>
<td>?</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Consumption of dietary fibers</td>
<td>-</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Consumption of unpasteurized milk</td>
<td>-</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Rural or low hygiene living</td>
<td>↓</td>
<td>↓↓</td>
<td>↓</td>
</tr>
<tr>
<td>Large or poor family</td>
<td>↓</td>
<td>↓↓</td>
<td>↓</td>
</tr>
<tr>
<td>Childhood pet contact</td>
<td>-</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Stress</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Smoking</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>History of infections</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Oral contraceptives</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Non steroidal anti-inflammatory drugs</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Appendectomy</td>
<td>↓</td>
<td>↑</td>
<td>-</td>
</tr>
</tbody>
</table>

Arrow up=increased risk, arrow down=decreased risk, 1maternal smoking during pregnancy (>10 cigarettes daily)

### 2 Juvenile idiopathic arthritis

#### 2.1 Diagnosis and classification

JIA is defined as arthritis of unknown etiology which starts before the age of 16 and persists for six weeks. For diagnosis, other causes of joint symptoms must be excluded. JIA is classified into seven subtypes which are presented in Table 4 (Petty RE 2004).
2.2 Etiology

The etiological triggering factor of JIA is unknown, but similar to other autoimmune diseases, it seems that both genetic as well as environmental factors play a part in disease onset. Studies of the environmental factors are rare and the results are not uniform. The most evidence is for the role of preceding infections such as streptococcus and virus infections for onset or flare-up of JIA (Table 3) (Ellis JA 2010). In a Finnish study which monitored familial aggregated JIA for 15 years, a concordance was 25% for monozygotic twins (Savolainen A 2000). To date, several human leukocyte antigen (HLA) polymorphisms have been linked to an increased or decreased risk of various JIA subtypes, as are other candidate genes such as genes coding for IL-6, macrophage migration inhibitory factor and natural resistance-associated macrophage protein 1 (Borchers AT 2006, Berntson L 2008).

Table 4  
Juvenile idiopathic arthritis (JIA) subtypes. Exclusions are: a.) psoriasis or history of psoriasis of the patient or the first degree relative, b.) HLA-B27 positive male having arthritis after age of six years, c) ankylosing spondylitis, enthesitis related arthritis, IBD related sacroiliitis, Reiter’s syndrome or acute anterior uveitis in first degree relative, d.) positive rheumatoid factor (RF) twice with three-month interval, e.) systemic JIA (Petty RE 2004)

<table>
<thead>
<tr>
<th>JIA subtype</th>
<th>Definition</th>
<th>Exclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Systemic arthritis</td>
<td>Arthritis with minimum 2 weeks fever, of which minimum 3 days daily, and at least 1 systemic manifestation: 1. nonfixed erythematous rash, 2. lymph node enlargement, 3. hepatomegaly / splenomegaly or 4. serositis.</td>
<td>a-d</td>
</tr>
<tr>
<td>2. Oligoarthritis</td>
<td>Arthritis in 1-4 joints during the first 6 months. Persistent form does not affect more than four joints throughout disease course while extended form affects more than four joints after six months.</td>
<td>a-e</td>
</tr>
<tr>
<td>3. Seronegative polyarthritis</td>
<td>Arthritis of 5 or more joints during the first 6 months. RF negative.</td>
<td>a-e</td>
</tr>
<tr>
<td>4. Seropositive polyarthritis</td>
<td>Arthritis of 5 or more joints during the first 6 months. At this time two positive tests for RF with 3-month interval.</td>
<td>a-c, e</td>
</tr>
<tr>
<td>5. Psoriatic arthritis</td>
<td>Arthritis combined with psoriasis and 2 of the following: 1. dactylitis, 2. nail pitting or onycholysis, 3. psoriasis of a first degree relative.</td>
<td>b-e</td>
</tr>
<tr>
<td>6. Enthesitis related arthritis</td>
<td>Arthritis and / or enthesitis with 2 of the following: 1. presence or history of inflammatory lumbosacral or sacroiliac joint pain, 2. positive HLA-B27, 3. male over 6 years of age, 4. symptomatic anterior uveitis, 5. first degree relative having history of ankylosing spondylitis, enthesitis related arthritis, IBD related sacroiliitis, Reiter’s syndrome or symptomatic anterior uveitis.</td>
<td>a, d, e</td>
</tr>
<tr>
<td>7. Undifferentiated arthritis</td>
<td>Arthritis that does not fit any of the categories listed above or fills the criteria of more than 1 category.</td>
<td>no</td>
</tr>
</tbody>
</table>

HLA= human leukocyte antigen, IBD= inflammatory bowel disease
3 Immunological aspects in IBD and JIA

The following chapters briefly summarize some basic concepts of the immune system, concentrating on the function of T-cells and the immunological features linked to the development of IBD and JIA.

3.1 Evoking the immune response

Innate immunity cells, dendritic cells (DCs), macrophages and neutrophils, express pathogen recognition receptors, for example TLRs (Medzhitov R 2000) and intra-cellular NOD-like receptors (Martinon F 2005). In addition to receptor mediated endocytosis, immature DCs in tissue can also take up pathogenic material by receptor independent macropinocytosis (Janeway CA 2005 a). The binding of microbe derived structures to TLRs leads to the activation of DCs and secretion of cytokines and chemokines (Medzhitov R 2000). Mature DCs present antigens bound to membrane glycoproteins called major histocompatibility complexes (MHC) to T-cells of adaptive immunity, which leads to T-cell activation. By controlling the antigen presentation, the innate immunity system has a central role in conducting the adaptive immunity response type and magnitude (Medzhitov R 2000).

3.2 T-cells

The hallmark of a mature T-cell is expression of T-cell receptor (TCR) and surface molecules, cluster of differentiation-3 (CD3), and either of CD4 (effector cells) or CD8 (cytotoxic cells). Effector T-cell differentiation depends on presented antigen and cytokine environment during the antigen presenting process (Janeway CA 2005 a).

Type 1 Th cells (Th1) secrete dominantly pro-inflammatory cytokine IFNγ, and activate CD8+ T-cells and macrophages in response to infection with intracellular microorganisms (Lazarevic V 2011). Th2 response is characteristic of asthmatic and allergic conditions or helminth infection. Th2 cells secrete cytokines IL-4, IL-5, IL-9 and IL-13, and can activate B-cell response (Okoye IS 2011).

During the last decade, a new helper T-cell subtype, Th17 cells, has been described (Harrington LE 2005). Th17 cells secrete IL-17 cytokines that enhance the inflammatory process by increasing local production of cyto- and chemokines, leading to increased leucocyte recruitment, expansion of myeloid cell lines, and enhanced T-cell activation (Aggarwal S 2002). There can be plasticity between Th17 and Th1 cells, and the balance of cytokines like transforming growth factor-beta (TGF-β), IL-23 and IL-12 has been reported to affect the cytokine profile secreted by Th17 cells; varying from purely IFNγ or IL-17 secreting Th-cells to Th17 cells capable of secreting combination of IL-17, IFNγ and IL-22 (Lee YK 2009). Th17 cells are typically induced in the mucosal surface as a defence against bacteria and fungi (Hue S 2006, Lee YK 2009). Th17 cells and induced Tregs are reported to share TGF-β dependent differentiation pathways, and the
differentiation to either of these cells can depend on the cytokine environment as IL-6 promotes the Th17, and all-trans retinoic acid promotes the Treg pathway (Lee YK 2009). Further, Treg specific transcription factor FOXP3 expression is reported to inhibit the retinoic acid receptor-related orphan receptor C2 (RORC2) a key transcription factor of Th17 cells (Lee YK 2009, Bene L 2011, Strober W 2011).

### 3.3 Regulatory T-cells

CD4+CD25+FOXP3+ regulatory T-cells (Tregs) are described as a unique effector cell line with a suppressive function (Itoh M 1999). Natural Tregs develop in the thymus as an individual T-cell population which acquires an antiproliferative (anergic) stage and suppressive function before entering into circulation (Itoh M 1999). Adaptive Tregs (iTregs) are described as cells that develop in periphery from CD25-CD4+ T-cells upon stimulation in the presence of TGF-β and IL-2 (Allan SE 2007, Horwitz DA 2008). Several molecules commonly expressed on Tregs are used for identification of these cells such as CD25, CD127, GITR, FOXP3, cytotoxic T lymphocyte associated antigen-4 (CTLA-4) and lymphocyte activation gene-3 (Corthay A 2009).

![Figure 1](functions_of_tregs_modified_from_corthay_a_2009.png)

**Figure 1** Functions of Tregs. Modified from Corthay A 2009.

In periphery, Tregs play a central role in maintaining self-tolerance and preventing autoimmunity (Sakaguchi S 2006) (Figure 1). Tregs are suggested to have several mechanisms of suppression including secretion of anti-inflammatory cytokines IL-10, TGFβ or IL-35, or driving effector-Th cells to apoptosis due to consumption of maintaining cytokines such as IL-2 (Boden EK 2008). Tregs isolated from IL-10 deficient...
mice were unable to prevent experimental colitis similar to wild-type mice Tregs (Asseman C 1999). Treg suppression can also be mediated by CTLA-4, a surface receptor that interacts with CD80 and CD86 expressed on antigen presenting cells (APCs) (Read S 2000, Sansom DM 2006). However, CTLA-4 seems not to be required for Treg function (Sansom DM 2006).

3.3.1 FOXP3

FOXP3 gene encodes transcription factor, originally called scurfin protein when discovered in mice (Hori S 2003). FOXP3 is expressed in CD4+CD25+ regulatory T-cells of mice and humans (Fontenot JD 2003, Hori S 2003). CD25 negative (CD4+CD25-) T-cells can also express FOXP3, although to a much lesser extent (Fontenot JD 2003, Hori S2003). FOXP3 is required for Treg development (Fontenot JD 2003). In a mice model, exogenous FOXP3 induced regulatory activity in CD4+CD25- T-cells during expression when cotransferred with retroviral infection-vector (Fontenot JD 2003, Hori S 2003). These ectopic FOXP3-transduced cells were able to inhibit experimental colitis of mice similarly to naturally occurring Treg cells (Hori S 2003). The importance of FOXP3 function in humans is underlined by the finding that the mutations affecting to FOXP3 function lead to a severe autoimmune syndrome called immune dysregulation, polyendocrinopathy autoimmune enteropathy X-linked syndrome (IPEX). The symptoms of IPEX arise in early infancy and include diabetes, eczema, enteropathy, hematological abnormalities, endocrinopathies or renal dysfunction (Ruemmele FM 2004).

3.3.2 GITR

GITR is gene coding for a TNF receptor family protein. GITR was originally found in murine T-cell hybridoma cultures, where dexamethasone (DEX) increased its expression approximately sixfold (Nocentini G 1997). Thereafter, it was found that GITR expression does not necessitate GC, but GITR is expressed in low levels in almost every lymphoid tissue, and its expression is upregulated on unselected T-cell activators such as anti-CD3 or Concaavallin A (Nocentini G 1997).

GITR has been suggested as an activity marker of Tregs (Corthay A 2009), although Treg activity does not necessitate functional GITR because mouse GITR+ and GITR-Tregs suppressed Th cells equally (Ronchetti S 2004). In mice, GITR is expressed in naïve Tregs, and its expression increases upon stimulation with anti-CD3 combined to IL-2 or anti-CD28 in mice (McHugh RS 2002, Shimizu J 2002, Kanamaru F 2004). GITR is also described at low level in resting mice Th cells, and is upregulated in these cells upon activation (McHugh RS 2002, Shimizu J 2002, Kanamaru F 2004). Despite a higher GITR expression, activated Th cells do not transform into a suppressive phenotype (Shimizu J 2002). GITR is a TCR co-receptor and enhances both Th and Treg cells (Kanamaru F 2004, Ronchetti S 2004). A combination of anti-GITR-antibody (anti-GITR-Ab) to anti-CD3 led to Treg proliferation in a dose-dependent manner (Kanamaru F 2004). In the
same study, activation of CD4+ T-cell cultures with either anti-GITR-Ab or anti-CD28 enhanced similarly and dose dependently the secretion of cytokines IL-2, IL-4, IFNγ and IL-10 (Kanamaru F 2004). GITR can also modulate T-cell apoptosis so that T-cell hybridoma cells that over-expressed GITR became resistant to anti-CD3 driven apoptosis (Nocentini G 1997).

GITR has been detected in several different immune cells such as natural killer-cells, B-cells, macrophages and DCs (Hanabuchi S 2006, Shimizu J 2002, Kanamaru F 2004). In vivo, GITR elimination, but also activation with anti-GITR-Ab led to development of autoimmune gastritis in mice (Shimizu J 2002).

3.4 Immunopathogenesis of IBD

3.4.1 Innate immunity mediated IBD pathogenesis

TLRs are conserved transmembrane receptors expressed on innate immunity cells such as DCs, macrophages and neutrophils, and also on epithelial and endothelial cells. TLRs recognize pathogen associated molecule patterns such as bacterial wall components (Medzhitov R 2000). TLRs enhance inflammation through an intra-cellular activation cascade resulting in activation of pro-inflammatory transcription factor, NFκB, and production of pro-inflammatory cytokines (Medzhitov R 2000, Martinon F 2005). Modified expression of TLRs in the gut has been suggested to be significant for IBD pathogenesis by leading to an altered response against normally tolerated luminal antigens such as commensal flora (Baumgart DC 2007 a, Bene L 2011). TLR4 was upregulated in IBD patients’ intestinal epithelial cells in comparison with controls (Cario E 2000). In the same study, TLR3 was expressed on normal mucosa, but downregulated in mucosa of active CD patients (Cario E 2000). In another study, TLR4 was up-regulated in biopsies of both UC and CD patients, while TLR2 was up-regulated in ileal biopsies of UC patients (Frolova L 2008). CD14, which is an up-stream molecule in the TLR4 pathway, was also over-expressed in both UC and CD in comparison with controls (Frolova L 2008). A further pathogen recognition receptor, NOD2, was expressed in CD patients’ inflamed ileum (Ogura Y 2003). The significance of pathogen recognition receptors in immunopathogenesis of IBD was underlined by the finding of a genetic association between mutations in gene of NOD2 in chromosome 16 in CD (Hugot JP 2001).

Altered function of DCs can also affect to IBD immunopathogenesis due to the role of DCs in tolerance induction and antigen specific T-cell activation. Sorted DCs from CD patients’ intestinal specimens expressed increased number of TLR2 and TLR4 (Hart AL 2005). In the same study, DCs of CD patients inflamed biopsies over-expressed co-receptor CD40, considered as marking of mature DCs (Hart AL 2005). IBD patients were also reported to have a lower number of circulating immature DCs than the controls (Baumgart DC 2005).
3.4.2 T-cell response in IBD

Traditionally, CD has been considered as a Th1 mediated inflammatory disease, and CU as a Th2 mediated (or resembling) disease (Strober W 2011). This is based on findings that in vitro activated lamina propria CD4+T-cells of CD patients secreted Th1 cytokine IFNγ and IL-2, but the secretion of Th2 cytokines IL-4 and IL-5 was decreased in comparison with controls. In the same study, CU patients’ lamina propria cells secreted IL-5, but not another Th2 key cytokine IL-4, or IFNγ (Fuss IJ 1996). Later, increased mucosal secretion of multiple cytokines was reported in IBD such as IL-12 and IL-18 in CD, IL-13 in UC and TNF-α, IL-1β and IL-6 in both diseases (Baumgart DC 2007 a, Strober W 2011, Eastaff-Leung N 2010).

In recent years, IL-17 immunity has undergone intensive research in IBD. The mucosal expression of IL-17 and the number of circulating Th17 cells has been shown to have increased in adult patients with active IBD (Annunziato F 2007, Hölttä V 2008, Eastaff-Leung N 2010). In adult CD patients’ ileal biopsies, IL-17A, IL-6 and FOXP3 messenger ribonucleic acid (mRNA) expression was increased in comparison with controls regardless of the disease activity (Hölttä V 2008). In IBD, especially those Th17 cells that are maturing in an IL-23 environment and capable of secreting both IL-17A and IFNγ may contribute to the immunopathogenesis of the disease (Annunziato F 2007). A group of adult CD patients’ gut Th17 cells were shown to produce IFNγ, and were considered as Th17/Th1 cells. Both Th17 cells and Th17/Th1 cells were found to express either RORC2 or T-bet, supporting the idea of the plasticity between Th17 and Th1 cell lines (Annunziato F 2007, Lee YK 2009). Th17 and Th17/Th1 cells were suppressed less by Tregs than Th1 or Th2 cells (Annunziato F 2007).

Furthermore, the balance between Th17 cells and Tregs has risen as a potential determinant of IBD immunopathogenesis (Eastaff-Leung N 2010). The role of Tregs in IBD immunopathogenesis is based on mice models of experimental colitis. In a scurfy mice model, a mutation of FOXP3 gene was shown to lead to lethal colitis (Brunkow ME 2001). In humans, the IPEX syndrome arising from the mutations impairing the FOXP3 function is presented with severe diarrhea due to enteropathy (Ruemmele FM 2004). In immunodeficient colitis mice, a transfer of Tregs was shown to lead to resolution of diseased gut infiltrates (Mottet C 2003). In adult IBD, the number of peripheral Tregs was decreased in comparison with controls (Eastaff-Leung N 2010, Wang Y 2011). However, in mucosa of adult IBD patients, the number of Tregs and FOXP3 mRNA and protein expression was increased (Wang Y 2011). The imbalance between peripheral Treg/Th17 compared with normal subjects is reported in both CD and UC (Eastaff-Leung N 2010). A model of a network of effector T-cells and secreted cytokines in IBD is presented in Figure 2.
3.5 Immunopathogenesis of JIA

3.5.1 T-cell response in JIA

In JIA, the pathologically expanded synovial tissue and pannus are formed of proliferating synoviocytes and patches of lymphocytes and APCs, while the clonally expanded CD4+ T-cells are the most abundant cells of the synovium (Grom AA 1993, Grom AA 2000). In vivo, synovial CD4+ T-cells expressed surface molecules that are typical in an activated state (Wedderburn LR 1999, Grom AA 2000). The flow cytometry analysis of synovial fluid (SF) lymphocytes showed an over-expression of adhesion molecule recognizing, “primed” CD4+CD29 T-cells in SF in comparison with peripheral blood (Silverman ED 1993). In JIA patients, 5-15% of infiltrating synovial T-cells have been reported to express γδ-TCR suggesting recognition of mycobacterial antigens and stress-related heat shock protein 65 (Kjeldsen-Kragh J 1993, Grom AA 2000). Despite an activated phenotype, synovial T-cells are reported to have impaired potency for proliferation and activation in vitro (Grom AA 2000). In JIA, SF B-cells and CD4+ T-cells were decreased and cytotoxic CD8+ T-cells increased when compared with peripheral blood (Silverman ED 1993). This accumulation of CD8+ cells into joints suggests a difference from findings in adult
rheumatoid arthritis (RA), and characteristic for oligoarticular JIA (Wedderburn LR 1999).

In synovium of JIA patients, T-cell cytokine profile is polarized to Th1 type response. This has been shown in different JIA subtypes by comparing the IFNγ/IL-4 ratio of synovial T-cells with peripheral blood, and also by increased expression of Th1 linked chemokine receptors in synovial T-cells (Wedderburn LR 1999). JIA patients expressed TNF-α and TNF-β on synovial tissue (Grom AA 1996). It is likely that polarization toward Th1 response is not restricted to joints, as serum IL-12 measured with immunoassays was increased in all subtypes of active JIA in comparison with controls (Gattorno M 1998). During inactive stages, serum IL-12 was higher than in controls only in patients with systemic JIA (Gattorno M 1998). The patients with systemic JIA, but not other JIA subtypes, had elevated serum levels of IL-18, in comparison with controls (Maeno N 2002). The reports of other cytokines have not been uniform (Borchers AT 2006).

Two studies have explored the role of Th17 and Treg cells in JIA. In the first, SF IL-17 was higher than in controls, or in serum of the same patients (Agarwal S 2008). The levels of IL-17 in SF showed correlation with the clinical disease activity of the joints, but not with the measured inflammatory cytokines IL-6, IL-1β, TNF-α (Agarwal S 2008). Another study monitored the number of Tregs, and expression of FOXP3 and RORC2 in SF mononuclear cells (SFMC) and in PBMCs from patients with JIA (Olivito B 2009). JIA patients expressed lower number of Tregs in blood, but a higher number of CD4+CD25 bright T-cells in SF than normal controls. In the same study, RORC2 mRNA in SFMCs showed an inverse correlation with SFMCs FOXP3 mRNA. In addition, IL-17 secretion from SFMC cultures correlated inversely with the number of SF Tregs (Olivito B 2009). These studies demonstrate that reciprocal actions between Tregs and Th17 effector cells can have role in the pathogenesis of JIA.

3.5.2 Autoantibodies

Autoantibodies are not good diagnostic markers for JIA although antibodies, such as RF, antinuclear antibodies (ANA), anticardiolipin and antiphospholipid antibodies, are occasionally found in serum of JIA patients (Borchers AT 2006). RF, an immunoglobulin (Ig) that reacts with IgG Fc fragment, can be found in all five Ig isotypes. Reported numbers of RF positivity with tests in routine use in clinical laboratories vary between 2-21% depending on the JIA subtype (Borchers AT 2006). RF positivity is most common in polyarticular disease (Borchers AT 2006, Syed RH 2008). RF can be a predictor of severe disease course seen as radiological joint destruction and difficulty in achieving remission (Van Rossum M 2003 a, Van Rossum M 2003 b, Flatø B 2003.). ANAs can be detected in approximately 30-50% of JIA patients, most commonly in pauciarticular or oligoarticular JIA (Serra CR 1999, Borchers AT 2006). The presence of ANAa increases the risk of uveitis (Kotaniemi K 2001) and longer duration of active disease, but not disability (Oen K 2003). Cyclic citrullinated peptide (CCP) antibodies, including antiperinuclear factor or anti-keratin antibodies, are targeted antibodies of citrullinated fillagrin, a protein that is not
expressed in joint cartilage, but during skin and esophagus epithelial cell differentiation.
Anti-CCP antibodies have been shown to have high disease specificity in RA, but in JIA, the presented numbers for IgG anti-CCP positive patients vary highly, ranging from 2 to 77%, with anti-CCP positivity being highest in patients with seropositive polyarthritis (Van Rossum M 2003 a, Syed RH 2008). In JIA, CCP positivity has been linked to radiological joint destruction (Van Rossum M 2003 a).

4 Matrix metalloproteinases, their inhibitors and neutrophil released enzymes

4.1 Basic concepts of MMPs and TIMPs

4.1.1 MMPs

In humans, 24 MMP genes code for 23 different neutral zinc metalloproteinases that cleave and remove the extracellular matrix (ECM). The majority of MMPs are extracellular proteins. MMP-1, MMP-2 and MMP-11 can also have intra-cellular functions. MMPs are grouped according to their structure and preferred substrates into collagenases (MMP-1 -8 -13 -18), stromelysins (MMP -3, -10 -11), gelatinases (MMP-2 and -9), matrilysins (MMP-7 and -26), membrane-type MMPS (MMP-14-17, -24, -25) and others (MMP-12, -19, -20, -21, -23, -27, -28) (Nagase H 2006).

MMP activity is low in normal tissue, and their expression is controlled by cytokines, growth factors, hormones and interaction between cells and cells to ECM (Nagase H 2006). Most MMPs are secreted as pro-MMPs that become activated by proteolytic removal of amino terminus or by oxidants or denaturing agents (von Lampe B 2000, Nagase H 2006). In addition, MMPs can activate each other, for example MMP-14 is an activator of MMP-2 (von Lampe B 2000). MMPs can act as sheddases and activate, by cleaving (solubilising), molecules on the surface of immune cells, which can result in both anti –and pro-inflammatory effects. For example, MMP-7 that can activate protelytically anti-bacterial peptide α-defensin in the gut, but also cleave pro-inflammatory cytokine TNF-α independent of TNF-α converting enzyme (Burke B 2004).

MMPs are regulated by controlled activation of their precursors and expression of their inhibitors such as TIMPs and α2M. The relationship between active MMPs to TIMPs is considered to determine ECM degrading activity (von Lampe B 2000). Impaired regulation ECM formation or degradation can play a part in pathogenesis of multiple diseases such as arthritis, chronic ulcers, cancers and cardiovascular diseases (Nagase H 2006).
4.1.2 TIMPs

TIMPs are MMPs’ major cellular inhibitors (Baker AH 2002). TIMPs are secreted proteins, but can on the cell surfaces bind to MMPs and affect and focalize their activity (Baker AH 2002). TIMP1-4 share a similar structure of N-and C-terminal domains (Nagase H 2006). In addition to inhibition of MMPs, TIMPS are suggested to affect cell growth, apoptosis, tumorigenesis and angiogenesis (Baker AH 2002).

4.2 MMPs, TIMPS, α2M, MPO and HNE in IBD

4.2.1 MMPs and TIMPs

Altered immunological response plays a part in the pathogenesis of IBD leading to increased expression of pro-inflammatory cytokines in the gut (Baumgart DC 2007 a, Strober W 2011). Cytokines TNF-α, IL-1β and IFNγ can induce MMP expression in fetal gut experiments, and colonic cell cultures (Pedersen G 2008). MMPs are found in intestinal ulcers (Saarialho-Kere UK 1996) and are suggested to serve as a link between T-cell mediated gut inflammation and GI ulcer formation (Pender SL 1997). Indeed, artificial MMP-inhibitor had potency in the treatment of mice dextran sulfate sodium induced colitis (Naito Y 2004). UC has been associated with single nucleotide polymorphism of genes coding for MMP-3, MMP-8, MMP-10, and MMP-14, but these results have not been confirmed in other studies (Morgan AR 2011). The central findings of MMPs and TIMPs in IBD are summarized in Table 5. Until now, there have been very few studies of MMP expression in pediatric IBD.

4.2.2 α2M

The non-specific inhibitor of endoproteinases, α2M, serves as the main plasma inhibitor of MMPs (Baker AH 2002). α2M is a tetrameric plasma glycoprotein that is synthesised mainly by liver hepatocytes, but also in other cells, for example macrophages (Baker AH 2002). During MMP inhibition, α2M binds covalently to MMPs (Baker AH 2002), after which the α2M-MMP complex becomes bound into receptor (low density lipoprotein related-protein-1) and endocytosed (Strickland DK 1990, Baker AH 2002). Previously, serum α2M level was found to be lower in adult IBD patients than in normal controls when detected with electrophoresis (Weeke B 1971), while the fecal α2M level in immunonephlometry was increased (Becker K 1999). Fecal α2M was higher in active IBD, and in CD patients correlated with Crohn’s Disease Activity Index (CDAI) (Becker K 1999).
**Table 5**  
Central findings of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in IBD patients’ gut tissue samples

<table>
<thead>
<tr>
<th>Research</th>
<th>Patients (n)</th>
<th>Method</th>
<th>Central findings in IBD</th>
<th>Correlations to clinical activity parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bailey CJ 1994</td>
<td>Adult IBD (11)</td>
<td>2</td>
<td>In CD MMP-9 was increased in inflammatory infiltrates, MMP-3 associated with tissue damage sites in CD and UC</td>
<td>-</td>
</tr>
<tr>
<td>Saarialho-Kere UK 1996</td>
<td>Adult IBD (12)</td>
<td>1,2</td>
<td>High MMP-1 signal in IBD samples</td>
<td>-</td>
</tr>
<tr>
<td>Vaalamo M 1998</td>
<td>Adult IBD (24)</td>
<td>1,2</td>
<td>MMP-10, MMP-12-13 and TIMP-3 were similarly over-expressed in UC and CD</td>
<td>-</td>
</tr>
<tr>
<td>Baugh MD 1999</td>
<td>Pediatric and adult IBD (29)</td>
<td>2,3,6</td>
<td>MMP-1-3 and MMP-9 were over-expressed in IBD patient samples, neutrophil MMP-9 most abundantly</td>
<td>-</td>
</tr>
<tr>
<td>Von Lampe B 2000</td>
<td>Adult IBD (55)</td>
<td>2,3,4</td>
<td>MMP-1-3, MMP-14 and TIMP-1 expression was increased in active mucosa samples</td>
<td>MMP-1 and MMP-3 correlated with histological score</td>
</tr>
<tr>
<td>Louis E 2000</td>
<td>Adult IBD (38)</td>
<td>5</td>
<td>MMP-3 and TIMP-1 secretion increased in samples of inflamed IBD mucosa</td>
<td>MMP-3 and TIMP-1 correlated with TNF-α, IL-1β, IL-6 and IL-10 secretion and to degree of inflammation</td>
</tr>
<tr>
<td>Matsuno K 2003</td>
<td>Adult UC (19)</td>
<td>2</td>
<td>MMP-1-3, MMP-7, MMP-9 and/or TIMP-1-2 were expressed in IBD samples</td>
<td>MMP-7 expression in epithelial cells correlated with degree of inflammation</td>
</tr>
<tr>
<td>Rath T 2006</td>
<td>Adult IBD (40)</td>
<td>4,5</td>
<td>MMP-2, MMP-7 and MMP-13 RNA expression, and MMP-2 and MMP-9 protein level was increased in IBD samples</td>
<td>MMP-7 and MMP-13 expression was increased in non-IBD adenomatous polyps</td>
</tr>
<tr>
<td>Meijer MJ 2007 a</td>
<td>Pediatric and adult IBD (142)</td>
<td>5</td>
<td>MMP-1-3 and MMP-9 in relation to TIMP-1-2, and MMP activity increased in inflamed IBD mucosa</td>
<td>MMPs or TIMPs expression did not correlate with sample fibrosis or development of fistulae</td>
</tr>
<tr>
<td>Pedersen G 2008</td>
<td>Adult IBD (19)</td>
<td>4,6</td>
<td>MMP-1, MMP-3, MMP-7, MMP-9-10 expression was increased in epithelial cells of inflamed mucosa</td>
<td>-</td>
</tr>
<tr>
<td>Mäkitalo L 2009</td>
<td>Adult CD (17)</td>
<td>2</td>
<td>MMP-9, MMP-26, TIMP-1 and TIMP-3 expression decreased during immunosuppressive therapies</td>
<td>MMP-9, MMP-26, TIMP-1 and TIMP-3 showed correlations to histological score, calprotectin and/or CDEIS</td>
</tr>
<tr>
<td>Mäkitalo L 2010</td>
<td>Pediatric IBD (33)</td>
<td>2</td>
<td>Epithelial MMP-10 and stromal TIMP-3 expression were increased in IBD samples, MMP-7 was greater in CD than in UC samples</td>
<td>-</td>
</tr>
</tbody>
</table>

Methodology: 1= In situ hybridization, 2= immunohistochemistry, 3= Western blotting, 4=PCR, 5=enzyme linked immunoassay, 6= functional assays of enzyme activity; CDEIS= Crohn’s Disease Endoscopic Index of Severity

29
4.2.3 MPO

MPO is an enzyme abundantly present in primary granules of myeloid cells such as neutrophils, monocytes and macrophages (Sangfelt P 2001). Normally, reactive oxygen metabolites are converted in a two-step manner. First, superoxide dismutases convert superoxide anion into hydrogen peroxide which is then neutralized to water by catalase or glutathione peroxidase. However, in inflammatory conditions like IBD, there is excessive hydrogen peroxide (Nathan CF 1987), which can be dealt by other reactions such as Haber-Weiss reaction in the presence of transition metals (Kehrer JP 2000), or alternative pathways such as MPO activity leading to formation of hypochlorous acid, a potential cause of oxidative tissue damage (Kruidenier L 2003).

High gut MPO levels are associated with IBD. MPO enzyme activity and expression was increased in gut biopsies of adult IBD patients, and higher in inflamed samples than in non-inflamed or control samples (Kruidenier L 2003, Meijer MJ 2007 a). MPO detected with immunoassay was increased 2-18 fold in sigmoid perfusion fluid of adult UC patients in comparison with healthy controls, and this increase associated with the increase of IL-8 and TNF-α in perfusion fluid (Raab Y 1993). In adult IBD patients’ gut specimens, MPO activity correlated with activity of MMP-1, MMP-3 and MMP-9 (Meijer MJ 2007 b). In line with this, fecal MPO expression detected with immunoassay was higher in adult IBD patients in comparison with healthy controls. In this study, the highest fecal MPO levels associated with UC and to CD with high Crohn’s Disease Endoscopic Index of Severity (CDEIS>200) (Peterson CG 2002). Fecal MPO is also reported to correlate with endoscopic score in adult UC patients (Peterson CG 2007).

4.2.4 HNE

HNE is a proteinase released from activated neutrophil azurophilic granules (Young RE 2004). Neutrophil elastase can affect leukocyte extravasation, cytokine release, phagocytosis and endothelial cell injury (Smedly LA 1986, Young RE 2004). High HNE levels detected with immunoassays were found in adult IBD patients in comparison with controls, and especially in CD patients or patients with highly active disease (Adeyemi EO 1985, Fischenbach W 1987, Gouni-Berthold I 1999). In adult UC patients with active disease, HNE enzyme activity was increased in both plasma and parallel gut biopsy samples (Morohoshi Y 2006). Also in adult IBD, fecal HNE complexed to proteinase inhibitor was higher than in controls and correlated with disease activity index in active UC and in active colonic CD (Adeyemi EO1992). IBD patients’ plasma HNE level correlated with CRP and CDAI (Adeyemi EO 1985). In a mice model with induced colitis, neutrophil elastase inhibitor,ONO-5046, presented with reduced colonic inflammation in autopsy (Morohoshi Y 2006).
5 Therapeutic options of IBD and JIA

IBD and JIA share similar treatment goals, which are induction and maintaining of remission, preventing destructive effects like joint damage in JIA, improvement of the quality of life, and maintaining normal growth and development in pediatric patients with minimal adverse effects (Hashkes PJ 2005, Wilson D 2010). Medical therapy of IBD and JIA include anti-inflammatory and immunomodulatory medication, often used in both diseases (Table 7) (Hashkes PJ 2005, Baumgart DC 2007 b, Wilson D 2010, Beukelman T 2011). Other therapeutic options for IBD are operations and nutritional therapy (Wilson D 2010, Bernstein CN 2010), and in JIA intra-articular GC injections, physiotherapy, occupational therapy, and operations (Hashkes PJ 2005, Beukelman T 2011). For the management of systemic and polyarthritic JIA not responding to other medication, autologous stem cell transplantation has been tested (Hashkes PJ 2005), as is also in severe CD (Duijvestein M 2010).

6 Glucocorticoids in IBD and JIA

The main human endogenous GC is cortisol that is synthesized in the adrenal cortex in a circadian and stress-related fashion (Shirtcliff EA 2011, Sapolsky RM 2000). GCs widely affect the human genome, and glucocorticoid receptor (GR) is expressed in nearly all tissues of the body (Janeway CA 2005 b). Synthetic GCs differ mainly with their receptor affinity and balance between anti-inflammatory and mineralocorticoid effect (Table 6).

The medical use of GCs began in 1948 when the first RA patient received cortisone acetate intra-muscularly (Hench PS 1949). For the treatment of IBD, GCs have been used since 1950 (Truelove SC 1954). In IBD, GC rarely leads to mucosal healing, which was seen in studies where clinical healing due to GCs was not associated with endoscopical or histological remission (Beattie RM 1996), or with normalization (<100 μg/g) of fecal calprotectin (Kolho KL 2006).

Table 6 Comparison of the effect of synthetic glucocorticoids. \(^1\) relative to hydrocortisone, \(^2\) relative to prednisolone (Järvinen A 2005).

<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>Anti-inflammatory effect(^1)</th>
<th>Mineral corticoid effect(^2)</th>
<th>Equivalent dose (mg)</th>
<th>Effective time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>1</td>
<td>2</td>
<td>20</td>
<td>8-12</td>
</tr>
<tr>
<td>Prednisone</td>
<td>2.7</td>
<td>1</td>
<td>5</td>
<td>12-36</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>12-36</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>12-36</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>24-48</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>30</td>
<td>0</td>
<td>0.75</td>
<td>36-54</td>
</tr>
</tbody>
</table>
### Table 7  
**Medical therapies in adult and pediatric inflammatory bowel disease (IBD) and juvenile idiopathic arthritis (JIA)** (Hashkes PJ 2005, Baumgart DC 2007 b, Wilson D 2010, Beukelman T 2011, Goebel JC 2011).

<table>
<thead>
<tr>
<th>Medicine type</th>
<th>Product</th>
<th>IBD</th>
<th>JIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-aminosalicylates and sulphasalazine</td>
<td>Sulphasalazine</td>
<td>Induction and maintenance of remission of mild to moderate IBD. Prevention of colorectal carcinoma.</td>
<td>Optional 1. line DMARD for enthesitis related JIA, 2. line DMARD for oligo –and polyarthritis</td>
</tr>
<tr>
<td></td>
<td>Mesalamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-steroidal anti-inflammatory drugs</td>
<td>Not indicated</td>
<td></td>
<td>1. line medicine for oligoarthritids and seronegative polyarthritids. If not disease modifying during 4-6 weeks used later for symptom relief and to control fever.</td>
</tr>
<tr>
<td>and coxibs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Systemic</td>
<td>Induction of remission in moderate to severe IBD</td>
<td>As bridging regimen at the start of DMARDs, induction of remission in systemic JIA</td>
</tr>
<tr>
<td></td>
<td>Local</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rectal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other immunosuppressants/DMARDs</td>
<td>Azathioprine and 6-mercaptopurine</td>
<td>1.line immunomodulators as maintenance therapy in moderate to severe IBD</td>
<td>Azathioprine as optional DMARD in uveitis</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
<td>2.line immunomodulator of moderate to severe CD</td>
<td>1.line immunomodulator in polyarthritis and extended oligoarthritis</td>
</tr>
<tr>
<td></td>
<td>Leflunomide</td>
<td>Not indicated</td>
<td>2. line immunomodulator of polyarthritis</td>
</tr>
<tr>
<td>Anti-TNF-α agents</td>
<td>Infliximab</td>
<td>Induction and maintaining of remission for moderate to severe adult and pediatric CD and adult UC</td>
<td>Active arthritis of ≤ 4 joints not responding to DMARDs in six months or in three months if features of poor prognosis.</td>
</tr>
<tr>
<td></td>
<td>Adalimumab</td>
<td>Induction and maintaining of remission for moderate to severe adult CD</td>
<td>Arthritis of ≥ 5 joints not responding to DMARD within three months is moderate or high disease activity and in six months if low disease activity</td>
</tr>
<tr>
<td></td>
<td>Etanercept</td>
<td>Not indicated</td>
<td></td>
</tr>
<tr>
<td>Other biologic agents</td>
<td>Abatacept, Rituximab, Anakinra</td>
<td>Not indicated</td>
<td>History of arthritis ≤ 5 joints with poor prognosis not responding to TNFα inhibitors. Rituximab secondary to abatacept. Anakinra in systemic JIA not responding to systemic glucocorticoids.</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Metronidazole, Ciprofloxacin</td>
<td>Acute or penetrating CD</td>
<td>Not indicated</td>
</tr>
<tr>
<td>Probiotics</td>
<td>Not indicated</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DMARD=disease modifying antirheumatic drugs, THA=triamcinolone hexacetonide, TNF=tumor necrosis factor
The use of GC therapy is complicated due to the fact that some patients do not respond to therapy, and can be considered as steroid resistant. In addition, some patients become steroid-dependent, with a flare-up of the symptoms shortly after GC tapering leading to prolonged GC therapy. Approximately 40-60% of the adult IBD patients on oral GCs have been reported as achieving remission, but 25-30% became steroid-dependent (Creed TJ 2007), and the number of steroid-dependent patients can be even higher in pediatric IBD patients (Hyams J 2006, Jakobsen C 2011) (Table 8).

Table 8 Recent studies of glucocorticoid (GC) response in pediatric Crohn’s disease (CD) and ulcerative colitic (UC). Short-term response is defined after one month and long-term response after one year systemic GC therapy unless otherwise stated.

<table>
<thead>
<tr>
<th>Study</th>
<th>Patients (n)</th>
<th>Short-term response</th>
<th>Long-term response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Remission</td>
<td>Partial response</td>
</tr>
<tr>
<td>Tung J 2006</td>
<td>Pediatric CD (26)/UC (14)</td>
<td>62/50</td>
<td>27/29</td>
</tr>
<tr>
<td>Hyams J 20061</td>
<td>Pediatric UC (62)</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>Markowitz J 20061</td>
<td>Pediatric CD (109)</td>
<td>60</td>
<td>24</td>
</tr>
<tr>
<td>Krupoves A 20112</td>
<td>Pediatric CD (364)</td>
<td>55</td>
<td>37</td>
</tr>
<tr>
<td>Jakobsen C 20113</td>
<td>Pediatric CD (87)/UC (77)</td>
<td>63/64</td>
<td>29/28</td>
</tr>
</tbody>
</table>

1 Short-term response defined at 90 days; 2 Long-term response at 180 days; 3 Prolonged response defined as remission for 30 days after therapy

It is thought that GC pharmacokinetics, for example impaired absorption from the inflamed gut, did not explain the differences in GC responsiveness in pediatric IBD (Faure C 1998). Several molecular mechanisms during GC-GR mediated gene reading process can modulate GC responsiveness. For example, GR expression and ligand binding or affinity, GR isoforms (GRα, GRβ), single nucleotide polymorphism (BcII) of GR gene and altered function of GC transmembrane glycoprotein pump (P-glycoprotein 170) have been studied regarding GC therapy response in IBD (Barnes PJ 2009, Sidoroff M 2011). Several other approaches such as clinical, tissue specific, bioassays and gene studies have tried to find a
method to predict GC therapy response in IBD (Sidoroff M 2011; Table 9) and other GC treated diseases such as asthma (Barnes PJ 2009, Leivo-Korpela S 2011).

GCs can present with adverse effects (Figure 3). Budesonide is a GC molecule with higher than 90% first-pass metabolism leading to low systemic bioavailability, and there are two oral preparations with controlled ileal release (Seow CH 2008, Vihinen MK 2008). A Cochrane review has shown budesonide to have fewer side-effects, but also to be less effective in induction of remission in CD than conventional GCs (Seow CH 2008). In pediatric patients, GC effect on bone mineral content and growth has been a particular concern (Boot AM 1998, Hääläinen H 2010). GC side-effects are mediated by GR transactivation, as in diabetes mellitus and glaucoma, or transrepression during suppression of the hypothalamic-pituitary-adrenal axis, or both as in osteoporosis (Schäcke H 2002, Buttgereit F 2005). Until now, there has been no laboratory test in clinical use to predict therapeutic response to GCs.

![Figure 3](image.png)

**Figure 3**  *Adverse reactions to glucocorticoids (Buttgereit F 2005). HPA = hypothalamic-pituitary-adrenal. Drawing by Elina Vanninen reproduced with permission from Papunet.*
### Table 9

**Examples of the studies monitoring glucocorticoid (GC) response in pediatric and adult inflammatory bowel disease (IBD)**

<table>
<thead>
<tr>
<th>Research</th>
<th>Patients (n)</th>
<th>Studied markers</th>
<th>Methods</th>
<th>Association to glucocorticoid therapeutic response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hearing SD 1999</td>
<td>Adult UC (18)</td>
<td>Inhibition of thymidine uptake</td>
<td>T-lymphocyte stimulation test; suppression of thymidine uptake due to DEX was measured</td>
<td>T-cells from steroid responders were suppressed more by DEX than T-cells from partial responders or treatment failures</td>
</tr>
<tr>
<td>Flood L 2001</td>
<td>Adult UC (21)</td>
<td>GR mRNA and MTIIa mRNA, cortisol</td>
<td>Low-dose ACTH test, mRNA quantitation with hybridization assay</td>
<td>Non-responding patients expressed more MTIIa on leukocytes before and their serum cortisol level decreased more during GC therapy compared with steroid responders</td>
</tr>
<tr>
<td>Löwenberg M 2006</td>
<td>Adult UC (9)</td>
<td>STAT-5</td>
<td>Immunohistochemistry of colon biopsies</td>
<td>Biopsies of GC-resistant patients expressed STAT-5 while the biopsies of GC responders did not</td>
</tr>
<tr>
<td>Vihinen MK 2008</td>
<td>Pediatric IBD (22)</td>
<td>GBA</td>
<td>Bioassay</td>
<td>No association between serum GBA and GC therapy response</td>
</tr>
<tr>
<td>Nakahara S 2008</td>
<td>Adult IBD (188)</td>
<td>Panel of single nucleotide polymorphisms related to steroid responsiveness</td>
<td>Genotyping and haplotype analysis</td>
<td>In CD patients polymorphism of SLC22A5 gene allele - associated to steroid resistance</td>
</tr>
<tr>
<td>Vihinen MK 2009</td>
<td>Pediatric IBD (19)</td>
<td>Adiponectin, leptin</td>
<td>Immunoassays</td>
<td>Patients with early appering GC related adverse effects had high serum adiponectin</td>
</tr>
<tr>
<td>Turner D 2010 a</td>
<td>Pediatric UC (50)</td>
<td>GBA</td>
<td>Bioassay</td>
<td>No association between serum GBA and GC therapy response</td>
</tr>
</tbody>
</table>

UC=ulcerative colitis, STAT=signal transducer and activator of transcription, GR=glucocorticoid receptor, MT=metallothionein GBA=glucocorticoid bioactivity, DEX=dexametasone, ACTH=adrenocorticotropic hormone
6.1 Intra-articular corticosteroid therapy in JIA

Intra-articular corticosteroid therapy (IACS) is effective in the relief of pain and limited range of motion. IACS are indicated either for oligoarticular JIA or for treatment of flare-ups of individual joints in other JIA subtypes in combination with disease modifying antirheumatic drugs (Bloom BJ 2011). All patients seem to respond clinically to IACS within 48 hours (Padeh S 1998, Bloom BJ 2011). In JIA, IACS are disease modifying which was seen as relieved joint effusion and pannus in magnetic resonance imaging seven weeks after the injection (Huppertz HI 1995). Approximately 70-90% of triamcinolone hexacetonide (THA) treated, and 40-50% of methylprednisolone (MP) treated patients maintained remission over six months (Honkanen VE 1993, Padeh S 1998, Bloom BJ 2011). THA can provide longer remission than triamcinolone acetonide (TA) (Zulian F 2003, Zulian F 2004). Most commonly reported adverse effects of IACS are local flare or subcutaneous atrophy in approximately 3% of injected patients (Padeh S 1998, Bloom BJ 2011). Cushinoid appearance was reported in a chart review in two of 61 patients (Bloom BJ 2011), as was also in a case report of two other JIA patients after TA injections (Kumar S 2004). To date, there has only been one study designed to explore the systemic effects during IACS in JIA. It showed that after IACS, endogenous cortisol production measured from saliva was suppressed for 10-30 days, and that circadian fashion of GC secretion was also disturbed (Huppertz HI 1997). In another study in RA, serum MP levels increased and cortisol level decreased in a dose dependent manner after intra-articular MP injection, and was restored to normal within one week (Armstrong RD 1981). In two pediatric arthritis patients that developed a Cushinoid appearance after intra-articular TA injection, TA was detectable in plasma and urine for five months after the injection, using mass spectrometry (Kumar S 2004).

6.2 Immunological effects of GCs

After absorption, GC diffuses to cytoplasm where it binds to GR. Ligand binding leads to activation of GR, quick release from chaperone complex and entrance of the GR-GC complex into the nucleus after which it either participates in activation of anti-inflammatory genes (Figure 4), or suppression of activated pro-inflammatory genes (Figure 5) (Barnes PJ 2009). GC leads to an increase of circulating leukocytes, mainly neutrophils, and a decrease of eosinophils (Zhang X 2000, Franchimont D 2004, Sbiera S 2011). GCs’ therapeutic effect is a result of a broad anti-inflammatory effect causing the attenuation of inflammation (Table 10) (Franchimont D 2004).
Figure 4  Activation of anti-inflammatory target genes by GCs. Modified from Barnes PJ 2009. GR=GC receptor, GRE= GC response element, HAT=histone acetyltransferase

Figure 5  GCs can switch off activated pro-inflammatory genes by interacting with co-repressor molecules that attenuate coactivator activity of proinflammatory transcription factors (NFkB or activator protein 1) and lead to decreased histone acetylation and down-regulation of gene reading. Modified from Barnes PJ 2009. NFkB= nuclear factor kappa β, IKKβ= inhibitor of NFkB kinase, HAT=histone acetyltransferase, HDAC2=histone deacetylase, *down-regulation

<table>
<thead>
<tr>
<th>Glucocorticoid therapy</th>
<th>Physiological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulation of IL-1, TNF-α, GM-CSF, IL-4, IL-5, IL-12 etc.</td>
<td>Down-regulation of inflammation</td>
</tr>
<tr>
<td>Down-regulation of NOS</td>
<td>Down-regulation of NO</td>
</tr>
<tr>
<td>Up-regulation of lipocortin 1 leading to down-regulation of phospholipase A2 and arachidonic acid</td>
<td>Down-regulation of prostaglandins, leukotrienes, platelet activating factor and cyclooxygenase activity</td>
</tr>
<tr>
<td>Down-regulation of adhesion molecules</td>
<td>Reduced leukocyte extravasation</td>
</tr>
<tr>
<td>Up-regulation of endonucleases</td>
<td>Induction of leukocyte and eosinophil apoptosis</td>
</tr>
</tbody>
</table>

IL=interleukin, TNF=tumor necrosis factor, GM-CSF=granulocyte-macrophage-colony stimulating factor, NO(S)= nitric oxide (synthase)

6.2.1 GC effects on cytokine profile

GCs have a suppressive effect on inflammatory cytokines (including both Th1 and Th2 class cytokines) (Table 10) (Franchimont D 2004, Janeway CA 2005 b). Non-specific cytokine suppression of GCs is likely to be a result of disturbed antigen presentation leading to impaired activation of T-cells. GC treated DCs were expressed in an immature phenotype with higher endocytotic activity but lower antigen presenting and cytokine secreting capacity than untreated DCs (Piemonti L 1999). A gene profiling study monitoring the effect of DEX on human PBMCs using microarray analysis, PCR and flow cytometry found that GC down-regulated MCHII and co-stimulatory molecules like CD40 on mature DCs, which could lead to impaired DC antigen presentation and T-cell activation (Galon J 2002). Further, the shift toward Th2 response can occur due to a modulated innate immunity response by GCs. Mainly secretion of Th1 response regulating IL-12 (Lazarevic V 2011) in monocytes and DCs is downregulated (Blotta MH 1997, Vicira PL 1998), and anti-inflammatory (IL-10, TGF-β) cytokines are up-regulated (Galon J 2002).

During the adaptive immunity response, the shift toward Th2 response is mediated by lack of IL-12. IL-12 receptor expression in human PBMC cultures was down-regulated by DEX. This down-regulation was independent from the cytokine environment (IL-4, IL-10, TGF-β), and led to impaired IL-12 binding (Wu CY 1998). However, down-regulation of IL-12 receptors was not confirmed in another study (Franchimont D 2000). Instead, when human T-lymphocytes and two commercial cell lines were cultured with DEX, DEX inhibited IL-12 induced phosphorylation of signal transducer and activator of transcription 4 (Stat4) mediating Th1 cytokine response, with no effect on phosphorylation of Stat6 that is linked to Th2 signaling (Franchimont D 2000). GCs can also have pro-inflammatory
effects such as an up-regulation of several pro-inflammatory receptor genes (IL-1RI, IL-8R and TNF-receptor) and down-regulation of the anti-inflammatory decoy receptor IL-1Ra gene (Galon J 2002).

6.2.2 GC effect on Tregs

GCs are effective medicines in various autoimmune diseases. This could be mediated by an enhanced action of Tregs. However, until now the findings of the effect of GCs on Tregs have been confusing. Two studies have explored the effect of GC therapy on normal humans. The first *in vitro* study found a decrease of Treg surface marker CTLA-4 when DEX was added in the culture of tetanus toxoid activated PBMCs (Xiang L 2011). Another study monitored the effect of a 14-day high dose GC therapy on patients with sudden hearing loss, and found only a modest increase in the number of circulating FOXP3+T-cells accompanied with a decrease in frequency of these cells in relation to the whole CD4+ T-cell population (Sbiera S 2011). An increase in the number of Tregs or expression of their activity markers during GC therapy has been shown in several clinical studies in different inflammatory or autoimmune disorders (Karagiannidis C 2004, Suárez A 2006, Braitch M 2009, Sbiera S 2011).

7 Anti-TNF-α agents in IBD

TNF-α antagonist agents, infliximab (IFX) and adalimumab were introduced for treatment of adult CD (Hanauer SB 2002, Hanauer SB 2006) and IFX for UC (Rutgeerts P 2005) at the end of the 20th century. IFX is also recommended in the guidelines for the management of steroid refractory pediatric UC (Turner D 2011), and small studies have been published of use of adalimumab in pediatric IBD (Wilson D 2010). IFX is a chimeric TNF-α antibody, and adalimumab a humanized IgG TNF-α antibody (Hanauer SB 2002, Hanauer SB 2006). Both IFX and adalimumab bind to TNF-α with high affinity. IFX binds to both soluble and transmembrane TNF-α, while adalimumab binds to soluble TNF-α (Hanauer SB 2006, Owczarek D 2009). IFX induces mucosal healing of endoscopic lesions in adult and pediatric CD patients (D'haens G 1999, Borelli O 2004). In pediatric CD patients, the weight and height Z scores increased 6 months after one to three IFX infusions (Borelli O 2004). Of adult CD patients with moderate CD, 58% responded to first IFX infusion (Hanauer SB 2002), and the responder rates in pediatric CD patients were similar or better (Borelli O 2004, Wilson D 2010). Of adult CD patients, 45% maintained remission for a year after IFX induction (Hanauer SB 2002), while a three month remission rate in pediatric CD patients was reported to be lower at 30% (Wilson D 2010). Rate of induction and maintenance of remission for one year with adalimumab in adult CD was 36% and 41% respectively (Hanauer SB 2006, Colombel JF 2007). Of adult CU patients, 69% responded to IFX therapy within eight weeks, and 45% maintained remission for one year (Rutgeerts P 2005). Serious side effects such as infections, malignancies, autoimmunity and liver toxicity have been associated with the use of anti-TNF-α agents in IBD patients.
(Stallmach A 2010, Wilson D 2010). Compared with the other immunosuppressants, the risk of side-effects is not increased when anti-TNF-α agents are used alone, but the risk increases in a combination treatment with GCs or other immunosuppressants (Stallmach A 2010).

7.1 Immunological effect of anti-TNF-α agents

TNF-α is a pro-inflammatory cytokine secreted by monocytes, macrophages and T-cells (Van Deventer 1997). In three studies with pediatric patients with active IBD, increased level of TNF-α protein or immunostaining was found in feces, mucosal amina propria and serum (Braegger CP 1992, Murch SH 1993, Murch SH 1991). TNF-α has been reported to serve as a “downstream” activator of other proinflammatory cytokines, but also to enhance “upstream” production of IL-12 (Strober W 2011), thus the immunosuppressive effect of anti-TNF-α agent is extended beyond the down-regulation of TNF-α.

In adult CD, IFX down-regulated IL-6 and up-regulated inactive TNF-α in serum, but had no effect on PBMC capacity to secrete IFNγ or TNF-α upon in vitro activation (Cornillie F 2001). The decrease in the number of lamina propria mononuclear cells secreting IFNγ or TNF-α after IFX treatment ex vivo was demonstrated in two studies with adult CD patients (Plevy SE 1997, Cornillie F 2001). One study monitored the IFX effect in vivo to sorted DCs, and found that T-cell co-activator CD-40 on DCs was down-regulated with no correlation to inflammatory activity in the gut biopsies (Hart AL 2005). Recently, three studies reported a restored function of Tregs to mediate anti-TNF-α agent effect in adult IBD (Li Z 2010, Boschetti G 2011, Veltkamp C 2011). The number of blood CD4+FOXP3+ T-cells (both CD25+ and CD25-) and FOXP3 expression detected with flow cytometry increased two weeks after the start of anti-TNF-α therapy (Li Z 2010, Boschetti G 2011), and this increase in Tregs associated with a higher suppressive effect on activated Th-cells (Boschetti G 2011). In the third study, the number of circulating Tregs was decreased before IFX therapy in CD patients in comparison with the controls, and enhanced apoptosis was suggested to be the reason based on annexin V staining. Increased apoptosis was also detected in lamina propria of CD patients (Veltkamp C 2011). In the same study, IFX was shown to restore peripheral Treg cells by normalizing apoptosis with simultaneous decrease of serum caspase-3/-7 activity (Veltkamp C 2011). These findings of an anti-TNF-α therapy effect as restoring Tregs supressive capacity is in line with a previous report with RA (Ehrenstein 2004).

7.2 Predicting therapeutic response to anti-TNF-α agents in IBD

CRP and fecal calprotectin have been studied as markers for predicting therapeutic response to anti-TNF-α agents. CRP is an acute phase protein secreted by liver hepatocytes upon inflammatory activation with IL-6, TNF-α and IL-1β (Vermeire S 2006). Two studies with adult CD patients at IFX induction resulted in different outcomes of the CRP predictive value for the anti-TNF-α response. The first study with 226 patients
found the therapy responding patients to have higher baseline CRP than non-responding patients (Louis E 2002), while the other study with 279 patients did not find a significant difference in baseline CRP level regarding the therapy response (Ester N 2002). The use of CRP as a prognostic marker to anti-TNF-α therapy in IBD is also restricted because CRP does not rise similarly in UC and CD patients (Vermeire S 2006).

Calprotectin and lactoferrin are neutrophil released proteins that are secreted in feces and measurable in IBD (Roseth AG 1999, Kayazawa M 2002). In adult CD patients, during three months of anti-TNF-α therapy, the decrease of fecal calprotectin and lactoferrin correlated with the decrease of CDEIS (Sipponen T 2008 b). In another study with adult CD patients, the decrease of fecal calprotectin correlated with the decrease of high sensitive CRP one week after the first IFX infusion, but not with the decrease of the Harvey Bradshaw index of disease activity (Lönnkvist MH 2011).

There were no studies to be found exploring markers to evaluate anti-TNF-α therapeutic response in pediatric IBD. Several serological markers have been studied to monitor the effect of anti-TNF-α therapy in adults, but these have not been shown to be superior to CRP or fecal calprotectin (Table 11). Studies exploring immunological pathways during anti-TNF-α therapy are also presented in Table 11.
<table>
<thead>
<tr>
<th>Research</th>
<th>Diagnosis (n)</th>
<th>Tissue</th>
<th>Studied markers</th>
<th>Methods</th>
<th>Predictive value to anti-TNF-α therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nikolaus S 2000</td>
<td>CD (24)</td>
<td>Whole blood cultivation supernatants, gut</td>
<td>TNF-α, NFκB</td>
<td>Immunoassay, Western blot</td>
<td>Increase of NFκB in biopsies or TNF-α secretion in supernatants preceeded relapse</td>
</tr>
<tr>
<td>Esters N 2002</td>
<td>CD (279)</td>
<td>Serum</td>
<td>ASCA and pANCA</td>
<td>Immunoassay, immunofluorescence</td>
<td>ASCA or pANCA did not predict infliximab response</td>
</tr>
<tr>
<td>Gao Q 2007</td>
<td>CD (83)</td>
<td>Blood, serum, gut</td>
<td>MMP-2, MMP-9</td>
<td>Immunohistochemistry, immunoassays, PCR</td>
<td>MMP-2 or MMP-9 expression in the gut or blood did not associate to infliximab response</td>
</tr>
<tr>
<td>Mäkitalo L 2009</td>
<td>CD (12 with infliximab)</td>
<td>Gut</td>
<td>MMP-1, MMP-7, MMP-9-10, MMP-26, TIMP-1, TIMP-3</td>
<td>Immunohistochemistry</td>
<td>Of therapy responding markers, stromal MMP-9, MMP-26 and TIMP-1 correlated with histological score and MMP-26, TIMP-1 and TIMP-3 with fecal calprotectin</td>
</tr>
<tr>
<td>Li Z 2010</td>
<td>CD (23), UC (17)</td>
<td>Blood, gut</td>
<td>Treg cells, CD25 mRNA, CD4 mRNA, FOXP3 (mRNA, protein)</td>
<td>Flow cytometry, PCR, immunohistochemistry</td>
<td>During maintenance, number of blood FOXP3 positive cells increased more and FOXP3 and CD25 expression in the gut decreased more in treatment reponders</td>
</tr>
<tr>
<td>Boschetti G 2011</td>
<td>CD (16), UC (9)</td>
<td>Blood</td>
<td>Tregs</td>
<td>Flow cytometry and suppression assay</td>
<td>No correlation was found between the change of Treg number and the clinical scores during anti-TNF-α therapy</td>
</tr>
<tr>
<td>Veltkamp C 2011</td>
<td>CD (32), UC (24)</td>
<td>Blood, serum, gut</td>
<td>Apoptotic Tregs, caspase-3 and -7</td>
<td>Immunohistochemistry, flow cytometry, luminescent substrate assay</td>
<td>Blood Treg count increased and caspase -3/-7 activity decreased more in anti-TNF-α responding CD patients</td>
</tr>
<tr>
<td>Lönnkvist MH 2011</td>
<td>CD (22)</td>
<td>Serum, plasma</td>
<td>Calprotectin, total nitrite, suPAR, ghrelin, endothelin</td>
<td>Immunoassays, Griess reaction</td>
<td>The change of calprotectin and suPAR after infliximab induction correlated with the decrease of hs-CRP but not with clinical activity index</td>
</tr>
</tbody>
</table>

CD=Crohn’s disease, UC=ulcerative colitis, TNF=tumor necrosis factor, NF=nuclear factor, MMP=matrix metalloproteinase, TIMP=tissue inhibitor of MMPs, Treg=regulatory T-cell, CD25/4=cluster of differmetation 25/4, ASCA=anti-*Saccharomyces cerevisiae* antibodies, pANCA=perinuclear antineutrophil cytoplasmic antibodies, suPAR=soluble urokinase Plasminogen Activator Receptor, hs=high sensitive
Aims of the study

The ultimate aim of this thesis was to find new means to monitor the decline of the inflammation during GC and anti-TNF-α therapy, and to find new tools for recognizing patients who would benefit from these therapies.

The specific aims were:

I To investigate pediatric IBD patient’s serum induced immune responses on donor derived allogenic peripheral blood mononuclear cells during GC therapy.

II To study whether intra-articular GC injections have systemic effects that would reflect glucocorticoid bioactivity or patient serum immune activation potency in JIA patients.

III To study whether pre-treatment serum immune activation potency in adult CD patients is associated with treatment response during anti-TNF-α-therapy.

IV To monitor the effect of GC and anti-TNF-α-therapy on serum levels of MMPs and their inhibitors in pediatric IBD patients.
Study subjects and methods

1 Study subjects and samples

The summary of patient characteristics is presented in Table 12, and the order of the sample collection is shown in Figure 6. The samples were collected in sodium-heparine tubes. Serum for GBA measurements were collected between 8-11 am. After collection, all serum samples were stored at -20 or -70ºC. All patients or their parents had given their written consent. Study protocols were approved by the ethics committee of the Helsinki University Central hospital.

Table 12  The study subjects

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Number of subjects</th>
<th>Age (median, range)</th>
<th>Sex (f/m)</th>
<th>Diagnosis (n)</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBD children on GC therapy</td>
<td>19+19 (10 patients shared in I and IV)</td>
<td>13 (4-18)</td>
<td>6/13 in both studies</td>
<td>UC (16+11), CD (2+6), IC (1+2)</td>
<td>I+IV</td>
</tr>
<tr>
<td>IBD children on anti-TNF-α therapy</td>
<td>16</td>
<td>14 (8-18)</td>
<td>9/7</td>
<td>UC (5), CD (11)</td>
<td>IV</td>
</tr>
<tr>
<td>IBD children in remission</td>
<td>13</td>
<td>13 (9-18)</td>
<td>6/7</td>
<td>UC (6), CD (6), IC (1)</td>
<td>IV</td>
</tr>
<tr>
<td>Adult CD patients with anti-TNF-α therapy</td>
<td>15</td>
<td>25 (19-44)</td>
<td>6/9</td>
<td>CD</td>
<td>III</td>
</tr>
<tr>
<td>JIA patients with IACS</td>
<td>21</td>
<td>11 (7-17)</td>
<td>16/5</td>
<td>Oligoarthritis (13), seronegative polyarthritis (5), seropositive polyarthritis (1), psoriatic arthritis (1), undifferentiated arthritis (1)</td>
<td>II</td>
</tr>
<tr>
<td>Pediatric controls</td>
<td>19</td>
<td>12 (5-15)</td>
<td>8/11</td>
<td>Familial hypercholesterolemia (2), arthritis (1), healthy (16)</td>
<td>IV</td>
</tr>
</tbody>
</table>

IBD=inflammatory bowel disease, GC=glucocorticoid, TNF-α=tumor necrosis factor-alpha, JIA=juvenile idiopathic arthritis, IACS= intra-articular corticosteroid therapy, UC=ulcerative colitis, CD=Crohn's disease, IC=indetermined colitis
1.1 Pediatric IBD patients (I, IV)

Forty-four pediatric patients with active IBD were recruited for studies I and IV at the Childrens Hospital, University of Helsinki, Finland, between 2004 and 2010. Basic characteristics of the patients are presented in Table 12. The exclusion criterion for the GC treatment group was preceding systemic GC medication within one month. Patients in publication I have been described earlier (Vihinen MK 2008) (Table 12). GC treated patients had oral prednisolone with a median dose of 0.8 (IV) - 1 (I) mg/kg. Study IV included one patient with oral budesonide with 0.4mg/kg. Sixteen anti-TNF-α naïve patients were introduced to IFX 5mg/kg (IV). Concomitant medicines were used on a clinical basis and included 5-aminosalisylate products, azathioprine/methotrexate, antibiotics or oral GC therapy at the start of anti-TNF-α therapy, and maintained unchanged between the study samples. Patients were examined by pediatric gastroenterologists before, and two to four weeks after the start of the therapy. When available, ESR, CRP and fecal calprotectin were registred before and by the time of the
control visit. In study IV, a chart review was made to evaluate clinical disease activity with a three-step scale of physician’s global assessment (PGA) defined as 1=no or minor symptoms, 2=occasional diarrhea, pain or rectal bleeding and 3=diarrhea several times a day/night or daily rectal bleeding as described (Haapamäki J 2011).

1.2 Adult CD patients (III)

Characteristics of 15 adult CD patients from the Clinic of Gastroenterology, Helsinki University Central Hospital, Finland, have been described before (Sipponen T 2008b) (Table 12). IFX was given with a dose of 5mg/kg at week 0 and 8. One patient had subcutaneous adalimumab 80mg at week 0, and 40mg every other week until week eight. When clinically indicated, concomitant medication including 5-aminosalisylate products, azathioprine/methotrexate, antibiotics or GCs were given, but maintained unchanged between the study samples. Clinical disease activity graded with CDAI (Best WR 1976, Sipponen T 2008b) and endoscopic activity graded with CDEIS (Mary JY 1989) were evaluated before the therapies and at a three months control visit. CRP, ESR and fecal calprotectin were measured at the same time.

1.3 Patients with JIA (II)

Twenty-one outpatients with JIA from the Heinola Rheumatism Foundation Hospital (Heinola, Finland) entered the study with inclusion criteria of active joint inflammation with need of IACS, but not preceding GC injection within a month (Table 12). The number of injected joints (2-7) and the injected dose (sum GC dose median 2.5mg/kg) were judged on clinical grounds. IACS were performed either under local (n=4) or general anesthesia (n=15). All patients received systemic immunosuppressive and/or immunomodulating therapy on clinical grounds. The background medication was unchanged between the study samples. A pediatric rheumatologist examined the patients before injection, and at two months. During these visits, the number of joints with swelling, pain or limited range of motion was recorded, and patient and physician global assessments of the disease activity were evaluated, and Childhood Health Assessment Questionnaire (CHAQ) values were calculated (Pelkonen P 2001). Serum for GBA, cortisol and immunological assay was collected before the therapies, 7 (at 3pm) and 24 hours (at 7-8am) thereafter, and within a two months control visit (Figure 8). ESR and CRP were measured before IACS, and at a two months control visit.

1.4 Controls (IV)

The control group consisted of two different groups: pediatric IBD patients with inactive disease (n=13), and pediatric patients visiting hospital outpatient clinics for other reasons (n=19) (Table 12). PGA was determined from controls with IBD. There were no
statistically significant differences in any of the measured markers between the two control groups, thus the two control groups were united for analyses.

2 Laboratory methods

A summary of the used laboratory methodology is presented in Table 13.

2.1 T-cell stimulation assay for measurement of serum immune-activation potency

A new biological assay for detection of serum immune activation potency was developed in this thesis. The effect of the patient’s serum on activation markers of peripheral blood mononuclear cells (PBMCs) from an allogenic donor (referred to as target cells) was studied. The PBMCs were isolated from whole blood withdrawn from healthy donors. PBMCs were separated by Ficoll-Paque (Amersham Biosciences) gradient centrifugation (800G, 25min). The PBMC layer was washed three times with phosphate buffered saline (PBS) and centrifuged 10min with 400G between the washes.

For cell culturing, the target PBMCs were diluted in cell culture media to 2x10⁶cell/ml containing RPMI media with hepes buffer (Gibco), 2μM glutamine (Gibco) and 25μl/ml gentamycin (Gibco). Target cells, unstimulated or stimulated with phytohemagglutin (PHA) 5μg/ml, were cultured as duplicates in the presence of the IBD patient’s inactivated serum (56°C for 35 minutes) at a concentration of 8%, for 72 hours at 37°C in a humified atmosphere with 5% carbon dioxide. After the culture, the supernatants and the target cells were collected and centrifuged with 400G for 7 minutes. Lyses buffer (Sigma) was added on the target cells and the lysed cells, and the supernatants were stored at -70°C. During methodological testing, the target cell assay was repeated with two different healthy donors PBMCs.

2.2 Enzyme-linked immunoabsorbent assays (ELISAs)

2.2.1 Cytokine ELISAs

The concentration of IL-5 and IFNγ was studied with an in-house ELISA (Halminen M 1997) from the supernatants collected from the target cell cultures incubated with patient serum (see above). Detection was performed in duplicate wells. For the ELISA, flat-bottomed 96-well plates (Nunc) were coated 24 hours earlier with a cytokine-specific antibody (ENDOGEN monoclonal anti-human IFNγ or PharMingen anti-human IL-5) diluted in 0.1M Na₂HPO₄. The following day, the wells were washed with PBS/0.05% TWEEN, which was also used in the later washes. Then the filtered 1% bovine serum
albumin (BSA)-PBS was added, and after 30 minutes the wells were washed 3 times. The samples and the standards were diluted in 1% BSA-PBS/0.05% TWEEN (later the diluting buffer) and incubated on the plates for two hours. After washing the wells four times, the second antibody (ENDOGEN monoclonal biotinylated anti-human IFNγ or PharMingen purified anti-human IL-5) was added and incubated for 1.5 hours. After washing four times, Streptadivin-phosphatase (Zymed) with the diluting buffer (1:1000) was added and the wells were incubated for 30 minutes. The substrate Nitrophenylphosphate tabs (Sigma) in sterilized water (2mg/ml) was added, and the detection was performed with a Thermo Labsystems Multiscan Ascent. IL-17 was measured with an ELISA-kit according to the protocol set by the manufacturer (R&D Systems). In cytokine ELISAs, ΑΔ-value describing the effect of the PHA stimulation was calculated by subtracting the non-stimulated value from the stimulated value of every detected serum sample.

2.2.2 ELISAs for MMPs, TIMPs, α2M, HNE and MPO

Commercially available ELISAs according to the instructions of the manufacturer were used for analysis of total MMP-7 (R&D Systems), total MMP-8 (Medix Biochemica), total pro-MMP-9 (Amersham Biosciences), HNE (Bender MedSystems mbH), and MPO (Immunodiagnostic AG) as described earlier (Tuomainen AM 2007, Rautelin H 2009, Rautelin H 2010). Commercially available ELISAs according to the manufacturer’s instructions were also used for detection of TIMP-1 (GE Healthcare), TIMP-2 (GE Healthcare) and total α2M (Immunodiagnostic AG). The calculation for MMP-7-9/TIMP-1 and MMP-7-9/TIMP-2 ratios was performed as mol/L.

2.3 Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

FOXP3, GITR and IFNγ spesific mRNA were measured in donor derived PBMCs stimulated with patient serum as described above. In addition, IFNγ, IL-5, IL-10, FOXP3, GITR, GATA3, STAT-5b and t-bet specific mRNA were detected in PBMCs from pediatric IBD patients. Total RNA was isolated with GenElute Mammalian total RNA miniprep kit (Sigma) and RNA concentration was measured by spectrophotometer. For reverse transcription, Tagman Reverse Transcription reagents (Applied Biosystems) were used, combined with treatment of total RNA at 10 ng/μl with DNAse I (0.01U/μl) (Roche Diagnostics) for eliminating genomic deoxyribonucleic acid. Quantitative RT-PCR was performed by predesigned FAM –labelled TaqMan Gene Expression Assay reagents (Applied Biosystems) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems) in triplicate wells. Threshold cycle (Ct) indicates the number of PCR cycles at which the target molecule exceeds a predefined fluorescence threshold value. The difference value (∆Ct) is achieved by subtracting the housekeeping gene’s (18s) Ct value from that of the target gene’s. As an interassay standard was used, exogenous c- deoxyribonucleic acid collected from PHA stimulated PBMC. ∆∆Ct is the difference between the ∆Ct of the analyzed sample and ∆Ct of the calibrator. $2^{-\Delta\Delta Ct}$ then gives a
relative amount of the target gene in the analyzed sample compared with the calibrator. For presentations, the relative amount of target genes was multiplied by 1000.

2.4 Glucocorticoid bioactivity (GBA)

Bio-assay for GBA measurement was performed as duplicates as published (Raivio T 2002). GBA measurement is a recombinant cell assay in which 10μl of patient serum is incubated overnight with COS-1 cells that are transfected with human GR coding expression vector, ARIP3, nuclear receptor co-activator, and reporter gene (luciferase and galactosidase). The detection limit is <15.6 nM of cortisol equivalents. GBA is sensitive in detecting biological activity of different synthetic GCs (Raivio T 2002).

3 Statistics

SPSS versions 13.0-17.0 (SPSS inc.) were used for statistical analyses. Mann-Whitney U-test was used for comparisons of groups of independent samples and Wilcoxon rank test was used for comparisons of pairwise groups of samples. Kruskall-Wallis test was used for non-parametric comparison of more than two groups with Dunn´s correction when appropriate. Correlations were calculated with Spearman´s rank order correlation test. P value < 0.05 was set for the level of statistical significance.
### Table 13  
**Applied laboratory methodology**

<table>
<thead>
<tr>
<th>Aim or measured marker</th>
<th>Methodology</th>
<th>Performer</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separation of PBMCs from peripheral blood</td>
<td>Ficoll gradient centrifugation</td>
<td>Author</td>
<td>I-III</td>
</tr>
<tr>
<td>T-cell stimulation assay</td>
<td>T-cell stimulation assay (described detailed in text)</td>
<td>Author</td>
<td>I-III</td>
</tr>
<tr>
<td>Detection of IFNγ and IL-5 from supernatants</td>
<td>In-house ELISA</td>
<td>Author</td>
<td>I-III</td>
</tr>
<tr>
<td>Detection of IL-17 from supernatants</td>
<td>Commercially available ELISA</td>
<td>Author</td>
<td>III</td>
</tr>
<tr>
<td>mRNA of IFNγ, IL-5, IL-10, FOXP3, GITR, GATA3, STAT-5b and t-bet</td>
<td>RT-PCR</td>
<td>PhD Harri Salo at professor Outi Vaarala’s laboratory</td>
<td>I, III</td>
</tr>
<tr>
<td>GBA</td>
<td>Bioassay</td>
<td>Professor Olli A Jänne’s laboratory</td>
<td>II</td>
</tr>
<tr>
<td>MMP-7-9, TIMP-1-2, HNE, MPO and α2M</td>
<td>Commercially available ELISA</td>
<td>PhD, DDS Taina at professor Tervahartiala at professor Timo Sorsa’s laboratory</td>
<td>IV</td>
</tr>
<tr>
<td>Cortisol</td>
<td>Commercially available ELISA</td>
<td>Clinical laboratory</td>
<td>II</td>
</tr>
<tr>
<td>Fecal calprotectin</td>
<td>Commercially available ELISA</td>
<td>Clinical laboratory</td>
<td>I, III-IV</td>
</tr>
<tr>
<td>ESR, CRP</td>
<td>Routine protocol</td>
<td>Clinical laboratory</td>
<td>I-IV</td>
</tr>
</tbody>
</table>

PBMC= peripheral blood mononuclear cell, IFN= interferon, IL= interleukin, FOXP3= forkhead transcription factor 3, GITR= glucocorticoid-induced tumor necrosis factor receptor, STAT= signal transducer and activator of transcription, GBA= glucocorticoid bioactivity, MMP= matrix metalloproteinase, TIMP= tissue inhibitor of MMPs, HNE= human neutrophil elastase, MPO= myeloperoxidase, α2M= α₂-macroglobulin, ESR= erythrocyte sedimentation rate, CRP= C-reactive protein, ELISA= enzyme-linked immunoabsorbent assay, RT-PCR= reverse transcriptase-polymerase chain reaction
Results and discussion

1 Serum immune activation potency on target cells (I-III)

For this study, a new biological assay was developed with the aim to describe the individual sum effect of GC and anti-TNF-α therapies in patient serum samples. In this assay, naïve or activated PBMCs from healthy donors were stimulated in the presence of the patient’s serum, and up-regulation of cytokines and transcription factors were measured at protein level with ELISA, and at mRNA level with quantitative PCR. It is to be noted that the T-cell activation markers were not measured directly from patient serum or the patient’s immune cells, thus the results are not directly comparable with the immunological responses described earlier during GC or anti-TNFα therapy.

1.1 The assay development

The first pilot study was made by stimulating two different donors’ derived allogenic PBMCs (referred to as target cells) with pediatric IBD patients’ plasma withdrawn before and two to four weeks after the start of GC therapy. IFNγ secretion was median 75 000 pg/ml (range 53 000-93 000 pg/ml) before therapy, and median 28 000 pg/ml (range 53 000-93 000 pg/ml) after therapy (p=0.012; n=8; unpublished) when PHA activated PBMCs derived from a 31-year-old male were cultured with pediatric IBD patients’ plasma. IFNγ secretion was median 131 pg/ml (range 0-9500 pg/ml) before therapy, and median 0 pg/ml (range 0-9400 pg/ml; p=0.018; n=10; unpublished) when the same patient’s serum samples were cultured with PHA activated PBMCs derived from a 26-year-old female. This study was repeated with similar results (data not shown). The immunosuppressive effect mediated by GC treated patient serum was found regardless of the target cell responsiveness. It is to be noted that PBMCs derived from the female donor were not used in later experiments due to limited proliferation. The decision to continue to test the effect of patient serum instead of plasma was based on the easy availability of the serum samples. It was found that patient post-treatment serum suppressed PBMCs cytokine response similar to the plasma (below).

During the study protocol, there were several steps designed for avoiding technical bias. First, those serum samples that were compared with each other (before and after therapy) were always cultured on the same plates. The serum samples were pipeted on the plate’s minimum as duplicate wells, and the supernatants derived from the same serum sample were collected as a pool for analysis. For the third work (III), the assay protocol was modified to contain a control sample derived from one healthy individual on every cell culture plate. The in-house cytokine ELISA also contained a standard sample on each plate. The ELISA was performed as duplicates, and if the intra-assay variation became higher than 15% between the duplicated wells, the sample was re-analyzed. The final ELISA result represents the mean of the two analyzed wells.
The idea for this bioassay arose from three sources: first, from the knowledge that altered immunological response plays a major role in pathogenesis of IBD (Baumgart DC 2007 a); second, the finding that patient serum could be used in a bioassay to elicit a GR mediated response in COS-1 cell culture that was measurable with transactivation assay (GBA; Raivio T 2002); and third, the observation that in IBD, GC resistance reflected T-cell suppressive capacity, seen \textit{ex vivo} as PBMCs derived from GC resistant patients showed lower suppression of proliferation induced by increasing DEX concentration (Hearing SD 1999). Although not with the same protocol, the activation of peripheral blood lymphocytes with patient plasma has been previously described as an \textit{ex vivo} model of acute respiratory distress syndrome (Meduri GU 2002). As GCs widely affect the human genome, and multiple mechanisms related to GR and tissue responsiveness can mediate GC biological effects (Sidoroff M 2011), a bioassay, as presented here, could offer a promising tool for describing the individual sum effect of GC therapy.

\textbf{1.2 The effect of GC treated patient’s serum on target cell cytokine profile (I,II)}

IFN\textgreek{g} and IL-5 secretion from the target cells and changes in cytokine secretion during oral GC therapy and IACS are presented in Table 14. IL-5 secretion from unstimulated target cells was low or below the detection limit.

\textbf{1.2.1 Pediatric IBD patients with oral GC therapy (I)}

The median secretion of IFN\textgreek{g} induced by pediatric IBD patient serum from both unstimulated and PHA stimulated target cells decreased during a 2-4 week oral GC therapy (p=0.017 and p=0.006 respectively), and the trend was similar in IL-5 secretion in stimulated target cells (p=0.055) (Table 14). No correlations were found between weight related dose of GC and serum induced target cells IFN\textgreek{g} or IL-5 secretion, or with the change of these markers during the therapy (ΔIFN\textgreek{g}, ΔIL-5; all p=ns).
Table 14  Cytokine secretion from healthy donor derived peripheral blood mononuclear cells in the presence of serum withdrawn from pediatric inflammatory bowel disease (IBD) and juvenile idiopathic arthritis (JIA) patients at baseline and 2-4 weeks after the start of oral glucocorticoid (GC) therapy or 24 hours after receiving intra-articular corticosteroid therapy (IACS).

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Cytokine</th>
<th>Baseline (pg/ml; median, range)</th>
<th>After therapy (pg/ml; median, range)</th>
<th>Increase/Decrease/no change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children with IBD on oral GC (n=19)</td>
<td>IFNγ</td>
<td>0 (0-308)</td>
<td>0 (0-29)</td>
<td>1/7/11</td>
<td>p=0.017</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>91000 (7200-122000)</td>
<td>62000 (6600-122000)</td>
<td>4/13/2</td>
<td>p=0.006</td>
</tr>
<tr>
<td></td>
<td>IL-5 PHA</td>
<td>69 (0-148)</td>
<td>17 (0-164)</td>
<td>4/13/2</td>
<td>p=0.055</td>
</tr>
<tr>
<td>Children with JIA receiving IACS (n=21)</td>
<td>IFNγ</td>
<td>35 (0-95)</td>
<td>27 (0-75)</td>
<td>7/8/2</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>49000 (2900-16900)</td>
<td>52000 (122-152000)</td>
<td>6/10/0</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>IL-5 PHA</td>
<td>31 (2-144)</td>
<td>22 (0-135)</td>
<td>6/11/1</td>
<td>p=0.085</td>
</tr>
</tbody>
</table>

IFN=interferon, IL=interleukin, ns= p>0.05

The finding that patient serum induced changes in target T-cell responses during GC therapy is in line with a previous report that plasma from MP treated acute respiratory distress syndrome patients decreased TNF-α and IL-1β, and increased IL-10 expression in peripheral lymphocyte cultures detected with Western blot and PCR (Meduri GU 2002).

Here, the post-treatment serum mediated decrease in Th1 type cytokine IFNγ secretion from target cells, which is in line with the reported effect of GC as suppressing Th1 immunity (Franchimont D 2004). Interestingly, when the effect of GC therapy was studied as patients’ serum induced changes in IL-5 secretion from the target cells, a trend of decrease in IL-5 secretion was seen (Table 14). In the literature, the reported effect of GC therapy on Th2 cytokines such as IL-4, IL-5 and IL-10 is mainly enhancing (Franchimont D 2004). Enhanced Th2 response can result from concomitant Th1 suppression, especially lack of IL-12, demonstrated previously in vitro (Franchimont D 2004). Activated APCs treated with DEX showed a decreased capability to secrete IL-12, and in co-cultures these APCs increased IL-4 and IL-10 secretion from CD4+ T-cells compared with APCs not treated with DEX (Blotta MH 1997). GCs can have direct effects on Th2 transcription factors as DEX was shown to up-regulate Th2 transcription factor c-maf (Galon J 2002). The noted Th2 suppression could reflect GCs direct suppressive effect on lymphocytes. Previously, DEX was shown to inhibit T-cell proliferation in vitro in a dose-dependent manner (Hearing SD 1999). However, it is to be noted that here no GC was added to PBMC cultures, but instead PBMCs were treated with patient serum containing
therapeutic doses of metabolized GC. Opposing reports of GC mediated Th2 suppression also exist. *In vitro* IL-4 secretion and IL-4 mRNA expression decreased in activated human PBMC and purified T-cell cultures after adding physiological doses of hydrocortisone (Wu CY 1991). In line with this report are two reports on asthma: the first showing a significant decrease in IL-5 secretion after a single oral GC dose detected with immunoassay from PBMC culture supernatants (Majak P 2009); and the second reporting the decrease of IL-4 and IL-5 specific mRNA in cells of bronchoalveolar lavage fluid in steroid sensitive asthma patients (Leung DY 1995). The finding here, that GC treated patient serum showed a trend to decrease normal PBMCs IL-5 response, underlines GCs multidirectional effect and supports the concept of the benefit of GCs in the treatment of Th2 immune mediated diseases such as asthma or allergies.

In pediatric IBD patients, weight-related dose of achieved oral GC had no correlations with serum induced target cells IFN\(\gamma\), or IL-5 secretion from the target cells or Treg markers expression (reported below); thus the observed suppression of target cell responses was not solely due to exogenous GC concentration in serum, but reflects the net effect of individual inflammatory activity in the patient’s serum. It is suggested that in IBD, therapeutic response did not directly associate to dose of GC. This was based on the finding that no difference in therapeutic response could be associated with doses of intravenous GCs reaching equivalent plasma cortisol levels (Kaplan HP 1975). Further, the availability of biologically active GC did not associate with therapeutic response in pediatric IBD (Vihinen MK 2008).

1.2.2 JIA patients with IACS (II)

The total number of injected joints was 69 in 21 studied JIA patients. The doses in a single large joint (shoulder, knee or hip) ranged between 0.6-1.4mg/kg for MP, and 0.4-0.9mg/kg for THA. The median total sum dose of achieved GC (both MP and THA) of a single patient was 2.5mg/kg (range 1.3-7.4 mg/kg). 24 hours after IACS, the serum induced response on target cell IFN\(\gamma\) secretion was not seen (Table 14), and IL-5 secretion was marginally decreased (\(p=0.085\)). After IACS, the weight-related total GC dose showed an inverse correlation with post-treatment IFN\(\gamma\) and IL-5 secretion from activated target cells (\(r=-0.691\), \(p=0.003\) and \(r=-0.482\), \(p=0.043\) respectively), but not with the change of target cells’ cytokine secretion (\(\Delta\)IFN\(\gamma\) or \(\Delta\)IL-5; both \(p=ns\)).

The systemic GC treatment in IBD patients was seen as a serum mediated decrease in IFN\(\gamma\) and IL-5 responses in the target cells. However, in JIA patients, this kind of effect was only observed as a trend for IL-5 response after IACS, thus the systemic effect of IACS was not comparable with that seen during oral GC therapy. In RA, it is reported that after IACS the degree of GC systemic absorption was determined by the GC dose and by the area of inflamed synovial membrane (Armstrong RD 1981). The inflammation of the joint increases the joint permeability which can affect drug efflux (Middleton J 2004).
1.3 The effect of GC treated patient serum on target cell FOXP3 and GITR expression (I)

Serum withdrawn from pediatric IBD patients two to four weeks after the start of GC therapy induced lower levels of GITR specific mRNA expression in target cells when compared with the serum withdrawn at the start of treatment. A decrease in patient serum induced FOXP3 expression after GC therapy was seen in naïve, but not in activated target cells (Figure 7). Weight-related GC dose had no correlations with FOXP3 or GITR specific mRNA expression after two weeks GC therapy, or with the fold change of FOXP3 and GITR during therapy (all p=ns).

No previous reports were to be found describing GC effect on Treg markers FOXP3 or GITR in IBD. In a recent study in mice, DEX treatment decreased the number of circulating Tregs in vivo without effect on Tregs suppressive capacity in vitro (Sbiera S 2011). Similarly, GC therapy on allergic rhinitis down-regulated FOXP3 expression on nasal mucosal biopsies (Malmhäll C 2007). Two studies in asthmatic patients did not find changes in Treg expression after GC exposure. The study with 54 asthmatic children examined the effect of a single oral GC dose combined with regularly inhaled steroid therapy and showed no effect on Treg number or FOXP3 expression measured at three months (Majak P 2009). In another small study with adult asthmatic patients, no change was observed in Treg cell counts or GITR expression during four weeks inhaled GC therapy (Zhang Q 2008).

Here, the patient serum induced FOXP3 expression decreased at a level of statistical significance on naïve donor PBMCs, but the decrease of FOXP3 did not achieve the level
of significance on activated donor PBMCs. It has been previously suggested that the PBMC activation stage would affect GC response (Galon J 2002). In addition, healthy PBMCs have been suggested to respond differently to GC therapy than cell derived from diseased individuals (Galon 2002, Sbiera S 2011). Indeed, contrary to the findings here, the majority of *in vivo* studies have reported increased Treg numbers or FOXP3 expression after GC treatment in patients with asthma (Karagiannidis C 2004) or autoimmune disorders such as systemic lupus (Suarez A 2006) and multiple sclerosis (Braitch M 2009).

**1.4 Clinical implications of serum immune activation potency (II, III)**

**1.4.1 In JIA patients (II)**

Post-treatment clinical disease activity had several inverse correlations with target cell IFNγ and IL-5 secretion induced by patient serum withdrawn either before, or 24 hours after IACS (Table 15). No correlations were found between pre-treatment clinical disease activity and pre-treatment target cell cytokine secretion (all p=ns).

**Table 15**  *An inverse correlation between IFNγ and IL-5 secretion from phytohemagglutinin activated donor derived peripheral blood mononuclear cells cultured with patient serum withdrawn before and 24 hours after intra-articular corticosteroid therapy (IACS) and clinical picture assessed at two months.*

<table>
<thead>
<tr>
<th>Clinical picture at two months</th>
<th>Before IACS</th>
<th>After GC IACS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFNγ</td>
<td>IL-5</td>
</tr>
<tr>
<td>Number of active joints</td>
<td>ns</td>
<td>p=0.044</td>
</tr>
<tr>
<td></td>
<td>r=−0.479</td>
<td>r=−0.557</td>
</tr>
<tr>
<td>Number of joints with limited range of motion</td>
<td>ns</td>
<td>p=0.049</td>
</tr>
<tr>
<td></td>
<td>r=−0.471</td>
<td>r=−0.743</td>
</tr>
<tr>
<td>Number of swollen joints</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r=−0.660</td>
</tr>
<tr>
<td>CHAQ</td>
<td>p=0.030</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>r=−0.541</td>
<td></td>
</tr>
<tr>
<td>Patient’s global assessment</td>
<td>p=0.014</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>r=−0.582</td>
<td></td>
</tr>
<tr>
<td>Physician’s global assessment</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

IFN=interferon, IL=interleukin, CHAQ= Child Health Assessment Questionnaire, ns= p>0.05

Because an inverse correlation between clinical disease activity parameters and target cell cytokine secretion was seen both before and after IACS, this finding cannot solely reflect the differences caused by the exogenous GC which has leaked out from the joint. Contrary to the inverse correlations seen here between clinical disease activity and target cell
cytokine responses induced by patient serum, serum level of IL-12 protein was shown to be elevated in active JIA (Gattorno M 1998, Yilmaz M 2001), and correlated positively with ESR and CRP (Gattorno M 1998). In addition, in systemic and oligoarticular JIA, a positive correlation was found between serum IL-12 level and the number of swollen joints or the joints with limited range of motion (Yilmaz M 2001). The inverse correlation seen here as serum mediated impaired cytokine response in target cells could reflect the chronic inflammation induced immune suppressive state, or also the existence of negative feedback mediators in patient serum due to inflammatory activity of the patient.

1.4.2 Serum immune activation potency related to CDEIS and ESR in adult CD patients (III)

Before anti-TNF-α therapy, adult CD patient serum induced FOXP3 specific mRNA expression in target cells correlated inversely to CDEIS and ESR (r=-0.621, p=0.013 and r=-0.548, p=0.034 respectively). GITR specific mRNA expression correlated inversely with CDEIS (r=-0.625, p=0.013). The low target cells FOXP3 and GITR specific mRNA expression at baseline correlated with the beneficial change of CDEIS during three months anti-TNF-α therapy (r=0.600, p=0.018 and r=0.589, p=0.021 accordingly).

Tregs participate in controlling Th responses against pathogens of the gut, and their impaired function plays a part in IBD immunopathogenesis (Boden EK 2008). In adult IBD, the frequency of peripheral blood Tregs was decreased (Wang Y 2011, Boschetti G 2011) and there was an imbalance between expressions of circulating Treg/Th17 in comparison with controls (Eastaff-Leung N 2010). As Tregs suppress Th cells (Thornton AM 1998), it seemed logical that those patients whose serum mediated enhanced induction of Treg markers FOXP3 and GITR in vitro had clinically milder disease in vivo seen as low CDEIS and ESR. We did not measure Treg markers on the patient PBMCs, but it is possible that allogeneic PBMCs derived from healthy donors were capable of responding differently to patient serum than patient derived PBMCs in vivo.

Interestingly, the low FOXP3 and GITR specific mRNA expression in activated target cells at baseline associated with better treatment response during three months, seen as a decrease of CDEIS. Recently, anti-TNF-α therapy has been shown to restore the number and function of circulating Tregs in IBD (Li Z 2010, Boschetti G 2011, Veltkamp C 2011), previously shown also in RA (Ehrenstein MR 2004). These results suggest that there can be group of CD patients with low systemic Treg activity that may benefit from anti-TNF-α therapy induced Treg activation, but those with high Treg activity already before therapy would not benefit from further Treg activation achieved with biological therapy.

It is also possible that FOXP3 and GITR expression here reflected the activation of target cells rather than direct up-regulation of Treg activity (Allan SE 2007, Corthay A 2009). In this case, the high expression of FOXP3 and GITR is associated with impaired therapeutic response as similarly reported in adult RA patients in whom the high
inflammatory activity, seen as a high number of blood chemokine receptor -3 and -5 expressing CD4+ T-cells, or high serum IL-2 receptor level at the start of the anti-TNF-α therapy, associated with a worse therapeutic effect to anti-TNF-α agents (Nissinen R 2003, Kuuliala A 2006).

2 GBA (II)

2.1 The effect of IACS to serum GBA and cortisol

Serum GBA rose and cortisol decreased after the IACS; these changes were maintained 24 hours but were normalized within two months (Figure 8).

Endogenous GBA correlated with serum cortisol (r=0.821, p<0.001), and this correlation remained after IACS (r≥0.500, p≤0.058 for 7 and 24 hours and for 2 months). The weight-related total dose of injected GC correlated with GBA and its decrease (ΔGBA) within 24 hours (r=0.548, p=0.015 and r=0.618, p=0.005 respectively). The correlation between endogenous serum GBA and cortisol is identical to that previously reported (Raivio T 2002, Vihinen MK 2008). GBA rose and serum cortisol level decreased after IACS. Correspondingly, in RA, serum cortisol level decreased and MP level increased after intra-articular MP injection (Armstrong RD 1981). This GC leakage from the inflamed joint can cause systemic adverse effects on JIA patients. In a chart review of 61 patients, two patients (3%) with a Cushinoid appearance were found (Bloom BJ 2010). There is also a case report of two JIA patients having a Cushinoid appearance after TA injections (Kumar S 2004). There is one previous study designed to monitor the systemic effect of IACS in
JIA. After IACS, endogenous cortisol production measured from saliva was suppressed for 10-30 days and the circadian fashion of GC secretion was disturbed (Huppertz HI 1997). Taken together, the finding that GBA rose and cortisol decreased after IACS demonstrates that intra-articular GC delivery is evoking a systemic GC effect in the patient. This systemic GC effect further seemed to modulate the immune activation potency of the JIA patient’s serum (above).

2.2 Comparison between GBA and serum immune activation potency

At baseline, GBA correlated positively with potency of JIA patients’ serum to induce IFNγ secretion from activated target cells (r=0.503, p=0.047). Similar pre-treatment correlation was not seen in pediatric IBD patients (p=ns; unpublished). In JIA patients, the increase of GBA (ΔGBA) due to IACS associated with the decrease of serum induced IL-5 secretion (ΔIL-5; r=-0.772, p<0.001). Similarly during oral GC therapy in pediatric IBD patients, ΔGBA associated with the decrease of patient serum induced IFNγ secretion (ΔIFNγ; r=-0.710, p=0.001; unpublished) (both Figure 9).

Figure 9  A.) In juvenile idiopathic arthritis (JIA) patients the change of serum glucocorticoid bioactivity (ΔGBA) correlated inversely with the change of IL-5 within 24 hours of intra-articular corticosteroid therapy (r=-0.772, p<0.001). B.) In pediatric inflammatory bowel disease (IBD) patients’ ΔGBA correlated inversely with the change of target cells IFNγ secretion (r=-0.710, p=0.001; unpublished).

An assay of GBA measurement is GR dependent and inhibited by GR antagonist mifepristone (Raivio T 2002). As clear correlation was seen between serum immune activation potency and GBA, it can be concluded that the serum induced suppression in target PBMC responses were mainly mediated by GR, although no direct correlation was seen between target cell cytokine secretion or Treg marker expression and administered per oral prednisolon dose in pediatric IBD patients.
Above, in chapter 2.1, serum GBA was shown to correlate highly to serum cortisol. Cortisol is an anti-inflammatory hormone secreted upon stress, and its secretion is stimulated by cytokines such as IL-2, IL-6, IFNγ and TNF-α (Sapolsky RM 2000). Proinflammatory cytokine IL-1 directly increased cortisone levels in a rat model (Besedovsky H 1986). GC effect has been suggested to vary from activating (permissive) to suppressive actions depending on the level of the released hormone (Figure 10; Sapolsky RM 2000).

Figure 10  Model of glucocorticoids permissive (activating) and suppressive actions. GR=glucocorticoid receptor, MR=mineralocorticoid receptor. Modified from Sapolsky RM 2000.

Here, a similar model of GC action was found, as at baseline endogenous GBA correlated positively with JIA patient’s serum immune activation potency, but after administration of exogenous GC, a classical GC effect of immune suppression (Franchimont D 2004) was seen as a rise in serum GBA associated with a pronounced decrease in serum induced cytokine secretion in pediatric IBD and JIA patients (Figure 9).

2.3 Clinical implications

In JIA patients receiving IACS, no clinical correlations were found between GBA at any time point and measurements for clinical disease activity or the inflammatory parameters ESR and CRP (all p=ns; data not shown). This is in line with previous reports during oral GC therapy in pediatric IBD where GBA did not associate with the treatment effect or to side effects (Vihinen MK 2008, Turner D 2010 a). The upper limit of endogenous circulating GBA in children is defined as 118 nM cortisol equivalents (Vihinen MK
3 MMPs, their inhibitors and neutrophil released enzymes during GC and anti-TNF-α therapy (IV)

Matrix metalloproteinases are enhanced by pro-inflammatory cytokines and over-expressed in active IBD (Pedersen G 2008). Here the serum profile of total MMP-7, total MMP-8, total pro-MMP-9, TIMP-1 and -2, total α2M, HNE and MPO was studied in pediatric IBD patients’ serum at baseline and within a month after the start of oral GC therapy or at week two after the first IFX infusion.

3.1 Comparison between patients with active IBD and controls

Before therapies, all measured MMPs, MMP-7-9/TIMP-1 ratios, MMP-8-9/TIMP-2 ratios, α2M, HNE and MPO were higher in active IBD patients than in controls (all p≤0.022; Table 16). At baseline, TIMP-1 was higher than in controls only in those IBD patients with GC therapy (p=0.007) and MMP-7/TIMP-2 was higher in patients with anti-TNF-α therapy (p=0.036).

In line with the results here, unbound-plasma MMP-9 measured with gelatin zymography was increased in adult CD patients in comparison with controls (Kossakowska AE 1999). In mucosal biopsies, MMP-9 has been shown to be over-expressed in adult IBD in comparison with controls (Baugh MD 1999, Mäkitalo L 2009), but this finding was not consistent in pediatric IBD (Baugh MD 1999, Mäkitalo L 2010). In line with the finding of a higher TIMP-1 level in pediatric IBD patients (GC group) than in controls, plasma and serum level of TIMP-1 was shown to be higher in adult IBD patients compared with controls (Wiercinska-Drapalo A 2003, Kapsoritakis AN 2008, Wang YD 2009). Circulating TIMP-1 levels also correlated with clinical and endoscopic activity indices (Wiercinska-Drapalo A 2003) and to CRP (Wiercinska-Drapalo A 2003, Kapsoritakis AN 2008). In one study where unbound-plasma TIMP-1 was measured with zymography, no difference was seen in TIMP-1 between CD patients and controls, nor an association with the disease activity index (Kossakowska AE 1999). This discrepancy between the results might be related to the different form of the measured protein, for example total, free or complexed; this information is rarely given in the text. Here, the GC treatment group had higher pre-treatment PGA than the anti-TNF-α group (PGA median 3 and 2 respectively). The lower disease activity is probably the reason why TIMP-1 was not statistically different between the anti-TNF-α treatment group and the controls.

Contrary to the finding of high serum total α2M level in pediatric IBD patients, in adult IBD patients’ serum, α2M level was lower than in the controls when detected with electrophoresis (Weeke B 1971). It is possible that the differences in findings with serum
α2M level related to different disease activity between the studies, as 43% of the IBD patients in Weeke’s study had inactive disease, whereas in this study α2M level was compared between IBD patients with active disease to controls. Fecal α2M level detected with immunonephlometry was increased in IBD patients in comparison with controls and correlated with CDEIS (Becker K 1999).

Previously, in line with this study, HNE plasma level has been shown to be increased in adult IBD patients in comparison with controls and to be higher in active disease (Adeyemi EO 1985, Fischenbach W 1987, Gouni-Berthold I 1999, Morohoshi Y 2006). Similarly, the finding of an increased level of another enzyme found in neutrophils, MPO, in IBD patients’ serum in comparison with controls, was in line with a previous report of higher MPO level in adult UC patients’ serum than the normal level as reported by the manufacturer (Peterson CG 2007).

3.2 Changes in measured serum markers during oral GC and IFX therapy

In pediatric patients with active IBD two to four weeks after the start of oral GC therapy, the serum level of MMP-7, TIMP-1 and MMP-7/TIMP-2 ratio decreased (all p≤0.001) to the level of the controls, while MMP-9/TIMP-1 increased (p=0.0018). Although the decrease of serum α2M was clearly not significant (p=0.658), after one month GC therapy serum α2M level was comparable with the controls (p=0.185; Table 16). During GC therapy, there were no statistically significant changes in other measured MMPs, TIMP-2, the MMP/TIMP ratios, HNE or MPO (all p=ns). Two weeks after the first IFX infusion, serum α2M and HNE had increased (p=0.023 and p=0.026 respectively) and MMP-7 presented a trend of decrease (p=0.063). There were no statistically significant changes in other measured markers or MMP/TIMP ratios (all p=ns). Although the decrease of MMP-7/TIMP-2 during anti-TNF-α therapy was not statistically significant (p=0.438), after two weeks of the first anti-TNF-α infusion, MMP-7/TIMP-2 was no longer statistically different from the controls (p=120; Table 16).

No previous reports were found designed to monitor the effect of GC therapy on serum or tissue expression of MMPs or TIMPs in IBD. Here it was found that serum TIMP-1 level decreased within a month of the start of GC therapy. In line with this current study, the decrease in TIMP-1 level due to GCs has been previously demonstrated in vivo in asthmatic patients treated with inhaled and/or oral GCs that had lower TIMP-1 level in bronchoalveolar lavage fluid than the non-treated subjects (Mautino G 1999), and in vitro in bovine chondrocyte culture with lower TIMP mRNA expression and activity level after treatment with DEX (Sadowski T 2001). In addition, Cushing’s syndrome patients from cultured skin fibroblasts expressed TIMP-1 at a lower level than normal cells (Zervolea I 2005).
Table 16  MMPs, TIMPs, α2-macroglobulin (α2M), human neutrophil elastase (HNE) and myeloperoxidase (MPO) in pediatric inflammatory bowel disease patients' serum introduced to glucocorticoid or anti-TNF-α therapy. * p≤ 0.001 and **p<0.05 compared with controls.

<table>
<thead>
<tr>
<th>Biomarker; median (range)</th>
<th>Glucocorticoid</th>
<th>Anti-tumor necrosis factor-α</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow-Up</td>
<td>Baseline</td>
</tr>
<tr>
<td>MMP-7</td>
<td>2.9 (1.4-11)*</td>
<td>2.0 (1.3-5.8)</td>
<td>2.9 (1.7-8.6)*</td>
</tr>
<tr>
<td>MMP-8</td>
<td>213 (12-630)*</td>
<td>198 (9-872)*</td>
<td>83 (13-527)*</td>
</tr>
<tr>
<td>MMP-9</td>
<td>284 (13-867) *</td>
<td>379 (47-834) *</td>
<td>211 (34-964) *</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>127 (56-243)**</td>
<td>93 (41-153)</td>
<td>109 (79-226)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>142 (58-1310)</td>
<td>136 (95-538)</td>
<td>117 (67-588)</td>
</tr>
<tr>
<td>MMP-7/TIMP-1</td>
<td>0.02 (0.01-0.06)</td>
<td>0.02 (0.01-0.07)</td>
<td>0.03 (0.01-0.08)</td>
</tr>
<tr>
<td>MMP-7/TIMP-2</td>
<td>0.014 (0.01-0.04)</td>
<td>0.010 (0.01-0.03)</td>
<td>0.017 (0.004-0.05)**</td>
</tr>
<tr>
<td>MMP-8/TIMP-1</td>
<td>0.6 (0.05-2.2)*</td>
<td>0.9 (0.05-5.2)*</td>
<td>0.4 (0.03-2.7)**</td>
</tr>
<tr>
<td>MMP-8/TIMP-2</td>
<td>0.3 (0.02-1.35)*</td>
<td>0.4 (0.01-2.6)**</td>
<td>0.3 (0.01-1)**</td>
</tr>
<tr>
<td>MMP-9/TIMP-1</td>
<td>0.4 (0.03-3.0)**</td>
<td>1.1 (0.14-4.0) *</td>
<td>0.6 (0.06-3.7)**</td>
</tr>
<tr>
<td>MMP-9/TIMP-2</td>
<td>0.4 (0.03-2.0) *</td>
<td>0.4 (0.05-1.9) *</td>
<td>0.3 (0.06-1.9) *</td>
</tr>
<tr>
<td>α2M</td>
<td>4760 (2200-11300)**</td>
<td>3730 (2550-13000)</td>
<td>5250 (3590-11900)*</td>
</tr>
<tr>
<td>HNE</td>
<td>369 (160-2240)*</td>
<td>419 (89-2090)*</td>
<td>362 (110-2040)*</td>
</tr>
<tr>
<td>MPO</td>
<td>169 (110-570)*</td>
<td>211 (65-489)*</td>
<td>163 (76-503)**</td>
</tr>
</tbody>
</table>
The observed decrease in serum TIMP-1 could reflect the suppression of inflammation due to GCs with a concomitant decrease in the need of TIMP-1 mediated inhibition rather than a direct GC effect. In support of this assumption, DEX activated the TIMP-1 promoter area through GR in COS-1 cell cultures (Förster C 2007).

Also MMP-7 and MMP-7/TIMP-2 ratio decreased during GC therapy, but the decrease of MMP-7/TIMP-2 reflects the decrease of MMP-7, since TIMP-2 level remained constant during the therapy. In line with this observation, in human macrophage cultures, GC suppressed MMP-7 mRNA expression studied with Northern blot (Busiek DF 1995). A possible mechanism linking the decrease of MMP-7 to GCs therapeutic effect in IBD could be down-regulation of MMP-7 mediated proteolytic cleavage of innate immunity related molecules (Burke B 2004). MMP-7 is reported to proteolytically activate antimicrobial peptides such as α-defensin-5 expressed in Paneth cells of the intestine (Wilson CL 1999, Lopez-Boado YS 2000), but also to cleave TNF-α family members and pro-apoptotic Fas-ligand (Burke B 2004).

Within two weeks of the first IFX infusion, the serum level of α2M and HNE had increased. No human studies were found designed to monitor the effect of anti-TNF-α agents to these markers. Increase in serum α2M level during anti-TNF-α therapy seems reasonable since MMPs are over-expressed in IBD (Table 5), which has been suggested to contribute to IBD pathogenesis. As α2M is considered to serve as the MMPs major plasma inhibitor (Baker AH 2002), it is possible that increased α2M mediated suppression participates in the anti-TNF-α agents’ therapeutic effects in IBD.

The finding of increase of serum HNE level during anti-TNF-α therapy is rather confusing, as plasma and gut HNE activity was shown to be elevated in active IBD. Furthermore, in mice, intra-peritoneal delivery of NE-inhibitor had a therapeutic effect on treatment of induced colitis (Morohoshi Y 2006). The increase of serum HNE level during anti-TNF-α therapy could relate to increased apoptose of inflammatory cells reported to lead to HNE release (Vandivier RW 2002).

During anti-TNF-α therapy, serum MMP-7 level presented a trend of decrease. The anti-TNF-α treated patients were allowed to have GCs as concomitant medicines and had lower pre-treatment PGA than the patients at the start of GC therapy. This lower disease activity is probably the reason for not finding any significant decrease in serum MMP-7 level in this patient group similar to the GC treatment group. After the first IFX infusion, the MMP-7/TIMP-2 ratio resembled that of the controls. This may reflect the resolution of inflammation, as in the adult UC patients gut samples the high MMP-7/TIMP-2 ratio was associated with high histological activity in the gut specimens (Matsuno K 2003).

It is to be noted that here, IFX therapy showed no effect on serum pro-MMP-9 level. The only previous study monitoring the effect of anti-TNF-α therapy with ELISA on the serum MMP-9 level in adult CD patients with active disease found a decrease in serum MMP-9 just at the border of statistical significance in an in-house study, but no decrease was seen in a larger patient group (Gao Q 2007). Previously in adult CD, mucosal expression of MMP-9 decreased during anti-TNF-α therapy (Gao Q 2007, Mäkitalo L 2009). Also in an in vitro study, IFX down-regulated MMP-9 secretion into supernatants in intestinal tissue cultures (Meijer MJ 2007 b).
3.3 Clinical implications

At baseline, serum MMP-7 correlated with ESR in pediatric patients with active IBD (r=0.420, p=0.026, n=28). Also among all IBD patients, high serum MMP-7 associated with clinically active disease assessed with PGA (p=0.047, n=48). There were no other statistically significant correlations between measured pre-treatment serum markers and assessed clinical parameters in this study (above; all p=ns). During the GC therapy, the change of MMP-9/TIMP-1 (ΔMMP-9/TIMP-1) correlated positively with the decrease of ESR (ΔESR; r=0.543, p=0.045, n=14). No other correlations relative to changes in MMPs and clinical activity markers during GC or anti-TNF-α therapy were found (all p=ns).

Previously, several associations between MMP and TIMP expression in the intestinal mucosa samples of IBD patients and disease activity have been reported (Table 5). The studies of serum MMP levels are limited. In adult UC patients, the plasma level of TIMP-1 was associated with three-step disease activity (Wang YD 2009). No studies were found associating serum MMP-7 level to clinical disease activity in IBD, but the finding that MMP-7 level reflected the clinical disease activity is in line with reports at tissue level. In pediatric IBD patients, MMP-7 expression in colon samples was higher in CD than in UC samples. Five UC samples that were later re-diagnosed as CD presented higher MMP-7 and MMP-1 expression than the other UC samples, and MMP-7 expression was suggested to be useful in differentiating between UC and CD (Mäkitalo L 2010). MMP-7 expression in epithelial cells at wound edges in adult UC patients correlated with the inflammatory activity grade in these samples (Matsuno K 2003). Here, no direct correlations were seen between serum MMP-9 level and disease activity measurements. In adult CD patients’ gut biopsy samples, MMP-9 expression in neutrophils correlated with the histological score, CDEIS and CRP (Mäkitalo L 2009).

4 General discussion

4.1 Strengths and weaknesses of the study

The methodology for the assessment of the immunomodulatory effect of the patients’ serum was designed by the study group, and the clinical use of the method was analyzed in these studies, which could thus be called pilot studies.

4.1.1 Subjects and samples

This thesis is based on four individual studies, all of which included only 15 to 21 patients. The limited number of patients is a common problem in pediatric studies. During the sample collection, all consecutive pediatric IBD patients from the Helsinki University
Children’s Hospital (I, IV) and the Heinola Rheumatism Foundation Hospital (II) fulfilling inclusion criteria were assessed. The endoscopically evaluated adult CD patients’ samples were received as an extension of another study (Sipponen T 2008 b). Due to the small number of study subjects, no power calculations were made, thus the risk of a false-negative result is relatively high.

Studies I, II and III were designed to evaluate the effect of GC and anti-TNF-α therapy, and paired samples at different time points were considered to serve as reciprocal controls, thus no control group of healthy subjects were included in these studies. In retrospect, this is a clear weakness of these studies. In work IV, two control patient groups were included: patients with inactive IBD, and pediatric patients visiting Helsinki University Children’s Hospital for other reasons. In addition, the measurement of drug concentration in the study samples would have been useful to objectively evaluate the individual biological effect induced by patient serum on target PBMCs. With a lack of healthy controls and knowledge of exogenous GC concentration in the samples, the gained data must be considered as observational instead of explanatory.

In the last work (IV), it would have been informative to examine MMP expression in gut biopsies and MMP serum level as parallel samples, as the majority of previously published data on the role of MMPs in IBD concerns findings in the intestinal biopsies.

### 4.1.2 Pitfalls in biological assay for monitoring the treatment effects

A new cell culture assay was applied in studies I-III. The methodology is based on activation of PBMCs from healthy donors with PHA in the presence of patient serum. A panel of T-cell activation markers was measured from the supernatants and the target cells. The origin of target cells from different donors causes variation in the assay. Despite this, the serum mediated modulation of cytokine response of different target cells was repeated relatively well. For a character of a biological assay, the intra-assay variation must also be taken into consideration. To diminish the bias during the cell culture process, the same serum sample was cultured in minimum of the duplicate wells and supernatants and cells derived from one serum sample were collected into pools for analyses. During the ELISA, samples were analyzed in duplicates and the intra-assay variation limit between duplicated samples was set at 15%. The comparison of paired serum samples (pre-treatment and post-treatment) was always performed in the same assay, and thus the results should not be affected by the interassay variation.

### 4.1.3 Drug of choice in JIA

In work II, MP is the primary GC injected into the joints of the JIA patients. Of the IACS, THA is shown to be superior to other GCs and it is recommended as a first line GC to be used in intra-articular injections (Bloom BJ 2010, Beukelman T 2011). However, at the time of the sample collection 2004-2005, there were limitations in THA availability in Europe, affecting the drug of choice in the JIA patients.
4.2 Future prospective

Here a new biological assay based on activation of normal PBMCs in the presence of patient serum has been described. This assay might be useful in the evaluation of the systemic effect of GC therapy in patients. Measured cytokine secretion or transcription factor expression in donor derived PBMCs did not correlate to the dose of systemically administrated GC. Further, the activation level of donor PBMCs showed some correlations to clinical disease activity. These findings warrant further studies with larger patient groups to establish the methodology. This kind of assay could also be tested in other patient groups with allergic or autoimmune type diseases necessitating the use of GCs. Regarding the future development of the assay, testing with other types of cells, possibly from established and commercially available cell lines could be suggested. It is to be noted that the use and continuation of both GCs and anti-TNF-α agents is based on clinical need and patient response. Until now, the clinical evaluation and patient-centered parameters such as PUCAI in pediatric IBD have been the best ways to monitor the treatment effect (Turner D 2010 b).

As a future investigation line regarding the MMPs, a paired testing with serum and gut biopsies is recommended. In the gut biopsies’ MMP expression, some discrepancy between pediatric and adult IBD has been reported, thus studying of the effect of GC or anti-TNF-α on adult serum samples would be recommended.

As previously suggested, perhaps no single serum marker will be able to reflect the wide GC therapy effect (Sidoroff M 2011), thus development of a multivariable analysis of several of the most promising markers could be a reasonable future prospect in this area.
Conclusions

Glucocorticoids (GCs) and anti-tumor-necrosis-factor-α (anti-TNF-α) agents are anti-inflammatory medicines used for the treatment of inflammatory bowel diseases (IBD) and juvenile idiopathic arthritis (JIA). Only part of the IBD patients responds to GC or anti-TNF-α therapies. These medications can have adverse effects, and these have also been reported after intra-articular corticosteroid therapy (IACS). Until now, there has been no clinical test predicting therapeutic response to these medicines. With this aim, a new cell culture assay was developed. This assay is based on monitoring the patient’s serum induced responses in normal allogenic target white cells. Further, other experimental biomarkers such as glucocorticoid bioactivity (GBA), matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) were monitored during the GC and anti-TNF-α therapies.

In the first study (I), a new biological assay for measuring immune-activation potency in patient serum was presented. During GC therapy, the treatment effect was seen as a serum mediated decrease in target cell interferon-gamma secretion and forkhead transcription factor 3 (FOXP3) and glucocorticoid induced tumor necrosis factor receptor (GITR) expression. This effect was independent of the GC dose. It was concluded that the individual net effect of GC therapy could be studied by monitoring the patient serum induced target cell responses.

In the second study (II), it was found that in JIA patients, IACS led to a rise of serum GBA and a decrease of cortisol and a reduction in serum induced target cells interleukin-5 secretion. It was concluded that there was a systemic leakage of biologically active GC after IACS.

In the third study (III), serum immune-activation potency was studied in adult Crohn’s disease (CD) patients at the start of anti-TNF-α therapy. At baseline, the high FOXP3 and GITR expression in target cells associated with a low Crohn’s disease endoscopic index of severity (CDEIS) and erythrocyte sedimentation rate. Interestingly, low pre-treatment expression of FOXP3 and GITR associated with good treatment response seen as a decrease of CDEIS during three months. It was concluded that the target cells FOXP3 and GITR expression showed potency to reflect clinical disease activity in adult CD patients during anti-TNF-α therapy.

In the final study (IV), the serum levels of MMP-7-9, TIMP-1, α2-macroglobulin (α2M), human neutrophil elastase (HNE) and myeloperoxidase were found to be higher in pediatric IBD patients with active disease than in controls. During GC therapy, MMP-7 and TIMP-1 decreased. During anti-TNF-α therapy, α2M and HNE increased. It was concluded that active IBD reflected serum MMP levels. GC and anti-TNF-α therapy had different kind of effect to serum MMP profiles that possibly reflect the therapeutic mechanisms of these medicines.
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