

From DEPARTMENT OF MEDICINE
Karolinska Institutet, Stockholm, Sweden

INFLAMMATORY MARKERS FOR EVALUATION OF INFLAMMATORY BOWEL DISEASE THERAPY

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M Pharm Sc



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To my family

Nothing worth having comes easy

ABSTRACT

The lack of reliable biomarkers for predicting the clinical course or monitoring therapeutic outcome in inflammatory bowel disease is an indisputable problem. The aim of this study was to evaluate markers of inflammation during inflammatory bowel disease therapy.

Blood chemistry markers were evaluated along with clinical activity and quality of life assessment during infliximab therapy in Crohn's disease patients, both the immediate response (one week) and long-term effect (up to five years). A majority of the patients showed improvement in clinical activity and quality of life as well as in hemoglobin, albumin and C-reactive protein levels. Using Harvey-Bradshaw index and Short Health Scale, infliximab demonstrated a prompt effect on clinical activity and quality of life and patients showed a maintained responsiveness over years.

Systemic inflammatory markers were followed along with clinical activity in Crohn's disease patients undergoing the first year of infliximab therapy. Healthy subjects were used as controls. Clinical activity improved with each infusion. High-sensitive C-reactive protein, calprotectin and nitrite improved after at least one infusion. However, calprotectin, nitrite and soluble urokinase plasminogen activator receptor were elevated compared with healthy controls throughout the study, indicating a continuous subclinical inflammation.

Fecal calprotectin and C-reactive protein were compared with clinical activity index in patients with Crohn's disease and ulcerative colitis during infliximab induction therapy. Calprotectin decreased significantly in responders following induction therapy. Moreover, fecal calprotectin exceeding a cut-off of 221 $\mu\text{g/g}$ was associated with a flare-up during the following 24 weeks.

Assessment of luminal NO concentrations in acute colitis patients prior to and after three days of corticosteroid therapy onset showed that NO levels were higher both at baseline and day 3 in patients responding to therapy than in non-responders. Furthermore, NO could be used as a predictor of colectomy as study endpoint. Baseline NO level below 2239 ppb was significantly associated with colectomy within one month from onset of corticosteroid therapy.

LIST OF PUBLICATIONS

- I. **Lönnkvist M**, Befrits R, Lundberg JO, Lundahl J, Fagerberg UL, Hjortswang H, van Hage M, Hellström PM

Infliximab in clinical routine: experience with Crohn's disease and biomarkers of inflammation over 5 years.

Eur J Gastroenterol Hepatol, 2009;21:1168-76

- II. **Lönnkvist M**, Theodorsson E, Holst M, Ljung T, Hellström PM.

Blood chemistry markers for evaluation of inflammatory activity in Crohn's disease during infliximab therapy.

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- III. **Lönnkvist M**, Befrits R, Ljung T, Holst M, Hellström PM.

Fecal calprotectin predicts clinical outcome during infliximab induction therapy.

Manuscript (submitted)

- IV. **Lönnkvist M**, Holst M, Ljung T, Hellström PM.

Luminal nitric oxide as an early predictor of colectomy in corticosteroid-treated acute colitis.

Manuscript

LIST OF ABBREVIATIONS

| | |
|------------------------------|--|
| ASCA | Anti- <i>Saccharomyces cerevisiae</i> antibodies |
| CD | Crohn's disease |
| CDAI | Crohn's disease activity index |
| CDEIS | Crohn's disease endoscopic index of severity |
| CRP | C-reactive protein |
| DAI | Disease activity index |
| ELISA | Enzyme-linked immunosorbent assay |
| GCS | Glucocorticosteroids |
| HBi | Harvey-Bradshaw index |
| IBD | Inflammatory bowel disease |
| IL-6 | Interleukin-6 |
| iNOS | Inducible nitric oxide synthase |
| NIPA | Near infrared particle immunoassay |
| NO | Nitric oxide |
| NO ₂ ⁻ | Nitrite |
| NO ₃ ⁻ | Nitrate |
| NSAID | Non-steroidal anti-inflammatory drugs |
| RIA | Radioimmunoassay |
| SHS | Short health scale |
| suPAR | Soluble urokinase plasminogen activator receptor |
| TNF | Tumor necrosis factor |
| UC | Ulcerative colitis |
| uPAR | Urokinase-type plasminogen activator receptor |
| 5-ASA | 5-aminosalicylic acid |

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1 INTRODUCTION

1.1 INFLAMMATORY BOWEL DISEASE

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory disorders in the gastrointestinal tract. Both types are characterized by periods of active disease (flare, relapse) with quiescent periods (remission) in between (1, 2). The common name for CD and UC is inflammatory bowel disease (IBD). General IBD symptoms are diarrhea, sometimes with blood in the stool, abdominal pain and weight loss, along with fever and symptoms of systemic inflammation in the more severe cases (3, 4). Approximately 40% of the IBD patients develop extraintestinal disease manifestations (5).

CD may affect the total gastrointestinal tract from the oral cavity to the anus with transmural inflammation, which might lead to complications (e.g. stricturing or fistulizing disease) and as many as 50% of the CD patients require surgery within ten years from diagnosis (6, 7). The site of inflammation is often located to the terminal ileum, caecum or colon.

In UC, the inflammation is restricted to the colonic mucosa, typically with involvement of the rectum. Depending on the anatomic extent of the inflammation, UC is classified as proctitis, left-sided colitis (involving the sigmoid colon, with or without descending colon involvement), or pancolitis. The inflammatory activity in CD is per definition patchy with inflamed areas surrounded by unaffected mucosa, whereas in UC the inflammation is continuous. However, differentiating Crohn colitis from UC can be difficult and some of the patients end up in a classification described as "indeterminate colitis" (8). IBD diagnosis is based on clinical symptoms and laboratory, endoscopic, radiological and histological findings.

The mean age of CD diagnosis ranges from 33 to 45 years of age in a North-American review (9), whereas the mean age for diagnosis of UC appears five to ten years later (10). In Europe, age peaks of incidence were reported between 15 to 25 and 25 to 35 years of age for CD and UC, respectively (11). Chronic inflammation might lead to dysplasia and colorectal cancer (12, 13). The cancer risk depends critically on disease duration and extent of inflammation (14). The age of cancer diagnosis in the IBD population is much lower than for sporadic

colorectal cancer in the general population (13, 15). UC patients have a normal life expectancy whereas in CD patients it is slightly reduced (16, 17).

The IBD incidence increases along with industrial development and westernized lifestyle (18, 19). The prevalence is also increasing, partly due to a decrease in IBD mortality (20). There seems to be a multi-factorial etiology causing IBD development, involving immune response deficiency, epithelial breach, triggering agents (microorganisms) and to some extent genetic predisposition with susceptible genes (e.g. nucleotide binding oligomerisation domain 2 [NOD2] in CD) (21-24). Heredity seems to be a stronger etiological factor in CD than in UC (25). Smoking is a generally accepted risk factor in CD, whereas it seems to reduce the risk of developing UC (6, 26, 27).

1.2 IBD THERAPY

There is as yet no curative IBD treatment, focus lies on symptomatic amelioration aiming to induce and maintain remission. Pharmacological therapy depends on disease location and activity.

1.2.1 Crohn's disease therapy

Glucocorticosteroids (GCS) and immunomodulators represent the mainstay of pharmacological treatment. Sulphasalazine is effective in inducing remission in patients with Crohn colitis, but is limited by the sulpha-related intolerance occurring in some patients (28). Mild to moderate inflammation with involvement of terminal ileum or right colon can be treated with targeted GCS (budesonide). The immunosuppressor azathioprine with its metabolite 6-mercaptopurine is given to patients with defective response to GCS. However, azathioprine has a slow onset of action over months and should be given as maintenance rather than induction therapy. Patients with signs of sepsis, bacterial overgrowth or perianal fistulas are treated with antibiotics when indicated. In severe CD or response failure, anti-tumor necrosis factor therapy (anti-TNF) is indicated. Surgery is an alternative to medical therapy, applied in complicated situations such as fibrotic strictures, fistulas, perforation or failing pharmacological response (29).

1.2.2 Ulcerative colitis therapy

GCS or drugs based on 5-aminosalicylate (5-ASA) are indicated in mild flares (30). Proctitis or left-sided colitis is locally treated with suppositories or foam (31). Moderate flares require oral GCS, often together with increased dosing of 5-ASA as maintenance therapy. In moderate to severe UC, combination therapy with GCS, 5-ASA and azathioprine is indicated. Anti-TNF is added if the response to combination therapy is failing (32). Severe UC entails increased risk for toxic dilation; those patients might need to be hospitalized and treated with intravenous GCS. Surgical options should be considered at the third day (or earlier) of intravenous GCS therapy (33). Anti-TNF can be given as rescue therapy. Colectomy is generally indicated in pancolitis, dysplasia or cancer, or in life-threatening complications such as perforation, dilation or massive bleeding (34).

1.2.3 Biological therapy

Infliximab (Remicade®) was the first biological agent approved for IBD therapy, causing a paradigm shift within the field (35, 36). Infliximab is a chimeric monoclonal anti-TNF antibody possessing qualities in mucosal healing, inducing and maintaining remission and to a certain extent also in fistula closing (37, 38). Through binding to and hence neutralizing soluble and receptor-bound TNF, infliximab blocks the TNF ability from further involvement in the inflammatory cascade. Colombel *et al.* showed that in patients with moderate to severe CD, who had never been exposed to immunomodulators or biological therapy, combination therapy with azathioprine and infliximab is superior to monotherapy with either choice (39). Whether anti-TNF should be introduced as “last-choice” therapy (step-up strategy), or first choice as a more aggressive way to take control over the inflammation (top-down strategy) is debated (40). Hitherto, step-up strategy is the common preference in the treatment of CD. In UC patients, the step-up strategy is a valid regimen.

Infliximab can be given as bridging therapy to azathioprine, or as scheduled infusion therapy. Induction therapy is given as infusions week 0, 2 and 6 (5

mg/kg bodyweight) (36). Thereafter, patients receive infusions every eight weeks as maintenance therapy (41).

1.3 CLINICAL ACTIVITY

The Crohn's disease activity index (CDAI) was developed as a tool to monitor disease activity in CD (42). It is today used as gold standard in clinical trials but has a few limitations. Firstly, the patient must fill in a one-week diary to correctly estimate the clinical activity and secondly, there is no histological or endoscopic criteria incorporated in the index. The validated endoscopic activity indices Crohn's disease endoscopic index of severity (CDEIS) and Simple endoscopic score for Crohn's disease (SES-CD), has again no definition of remission (43, 44). Harvey and Bradshaw developed a simplified version of the CDAI, the Harvey-Bradshaw index (HBI), which has shown strong correlation with CDAI (45, 46) (Table I). HBI is based on five items and can rapidly be evaluated at clinical visits. Clinical response is evaluated as a decrease of ≥ 3 points in HBI, and clinical remission as a point of < 5 (46).

Table I. Harvey-Bradshaw Index

| | | |
|---------------------------------|---------------------|----------|
| General wellbeing | Very well | 0 |
| | Slightly below par | 1 |
| | Poor | 2 |
| | Very poor | 3 |
| | Terrible | 4 |
| Abdominal pain | None | 0 |
| | Mild | 1 |
| | Moderate | 2 |
| | Severe | 3 |
| Number of liquid stools per day | | 1 p/each |
| Abdominal mass | None | 0 |
| | Dubious | 1 |
| | Definite | 2 |
| | Definite and tender | 3 |
| Complications | | 1 p/each |

In the 1950's, Truelove and Witts created a UC activity index built on a three graded scale of disease activity which is still in use (47). Another commonly employed UC index is the disease activity index (DAI), as developed by Sutherland *et al.* (48) (Table II). DAI is nearly identical to the Mayo score, which was described by Schroeder *et al.* in 1987 (49, 50) (Table III). When the endoscopic part of the Mayo score is excluded, the index is referred to as the partial Mayo score. In the partial Mayo score, a therapeutic response is calculated as a decrease of ≥ 3 points and clinical remission as an absolute point of < 2.5 (50).

Table II. Disease Activity Index

| | | |
|--------------------------------------|---------------------------------|---|
| Stool frequency | Normal | 0 |
| | 1-2 stools/day more than normal | 1 |
| | 3-4 stools/day more than normal | 2 |
| | >4 stools/day more than normal | 3 |
| Rectal bleeding | None | 0 |
| | Streaks of blood | 1 |
| | Obvious blood | 2 |
| | Mostly blood | 3 |
| Mucosal appearance | Normal | 0 |
| | Mild friability | 1 |
| | Moderate friability | 2 |
| | Exudation, spontaneous bleeding | 3 |
| Physician's rate of disease activity | Normal | 0 |
| | Mild | 1 |
| | Moderate | 2 |
| | Severe | 3 |

Table III. Mayo Score

| | | |
|--|--|---|
| Number of stools | Normal | 0 |
| | 1-2 stools/day more than normal | 1 |
| | 3-4 stools/day more than normal | 2 |
| | >5 stools/day more than normal | 3 |
| Rectal bleeding | None | 0 |
| | Visible blood with stool less than half the time | 1 |
| | Visible blood with stool more than half the time | 2 |
| | Passing blood alone | 3 |
| Mucosal appearing at endoscopy | Normal or inactive disease | 0 |
| | Mild disease | 1 |
| | Moderate disease | 2 |
| | Severe disease | 3 |
| Physician's rating of disease activity | Normal | 0 |
| | Mild | 1 |
| | Moderate | 2 |
| | Severe | 3 |

1.4 QUALITY OF LIFE

The Short Health Scale (SHS) was developed by Hjortswang *et al.* as a tool to evaluate the subjective quality of life in IBD patients (51, 52). SHS comprises a four-dimension questionnaire including symptom-burden, social function, disease-related worry and general well-being (Table IV).

Table IV. Short Health Scale

| | | |
|--|------------|---------------------------------|
| How severe are the symptoms you suffer from your bowel disease? | | |
| No symptoms | 0 -----100 | Very severe symptoms |
| Do your bowel problems interfere with your activities in daily life? | | |
| Not at all | 0 -----100 | Interfere to a very high degree |
| How much worry does your bowel disease cause? | | |
| No worry | 0 -----100 | Constant worry |
| How is your general feeling of well-being? | | |
| Very good | 0 -----100 | Dreadful |

1.5 MARKERS OF INFLAMMATION

Clinical activity indices are valuable in disease assessment, but since they are partly subjective, the result might not reflect the true inflammatory activity efficiently enough. Endoscopy is time-consuming, expensive and unpleasant for the patient. Laboratory markers present an objective and minimally invasive way of measuring momentary inflammation and could thus facilitate discerning of risk patients.

1.5.1 Circulating markers

C-reactive protein (CRP) was first described in 1930 by Tillett and Francis (53). It is used as gold standard in evaluating inflammatory activity. CRP is an acute-phase protein synthesized by hepatocytes as a response to inflammatory processes, infections and stress among others. Under normal circumstances CRP is produced in low quantities (<1 mg/L) (54). During inflammation, interleukin-6 (IL-6), IL-1 β and TNF influence the production of CRP, reaching levels of 50-500 mg/L depending on the severity of inflammation (55). The half-life of CRP is short (19 h), resulting in a rapid decrease once the inflammation has ceased. It has shown to predict relapse in IBD and colectomy in severe UC, as well as correlation with disease activity in both diagnoses, however stronger in CD (56, 57). Prior to anti-TNF treatment onset, baseline CRP-levels >5 mg/L has shown

to be associated with a higher response frequency than patients with CRP <5 mg/L (58), similar results were recently confirmed by Jürgens *et al.* (59).

Anti-*Saccharomyces cerevisiae* antibodies (ASCA) was in 1988 shown by Main *et al.* to be present in high titres in CD patients (60). For this reason, they declared a theory of yeasts playing a possible role in CD etiology. Giaffer *et al.* found higher IgG-levels in patients with ileal CD and suggested this to reflect an increased intestinal permeability (61). ASCA in CD has shown to be stable over time and does not depend on disease activity or duration (62). Presence of ASCA has not shown to predict anti-TNF therapy outcome (63). ASCA is detected in 39-70% of CD patients and 20-25% in UC patients respectively, compared with 0-5% in healthy controls (60, 64, 65).

Calprotectin (S100A8/A9) was described in 1980 by Fagerhol *et al.* (66). Neutrophils, monocytes and reactive macrophages derive calprotectin, a protein with bacteriostatic and fungistatic properties (67). It is involved in intracellular signal transduction and exerts regulatory inflammatory functions (68). In various inflammatory conditions, fecal calprotectin has shown to increase multifold and can be used in IBD diagnosis and monitoring of IBD therapy (69-73). Calprotectin in blood has shown to correlate with disease activity in inflammatory arthritides such as rheumatoid arthritis and reactive arthritis (74-76). Serum calprotectin has also shown to be elevated in children with IBD compared to controls (77).

In biological fluids, nitric oxide (NO) has a very short half-life and is rapidly oxidized to nitrite (NO₂) and nitrate (NO₃). Previously, those compounds have been considered as stable end-products of NO. However, recent research suggests a reverse pathway in which nitrite and nitrate are reduced back to NO, functioning as NO backup supply when the enzymatic pathway is insufficient or malfunctioning, e.g. during hypoxic conditions. In 1994, Lundberg *et al.* and Benjamin *et al.* demonstrated stomach NO generation from inorganic nitrite reduction (78, 79). Those were the first reports confirming a nitric oxide synthase (NOS)-independent source of NO. Moreover, Jansson *et al.* has shown that dietary nitrate protects against gastric ulcers derived from non-steroid anti-inflammatory (NSAID) drugs in the rat (80). This protection is eliminated after addition of topical antibacterial mouthwash, hence, the bacteria in the oral cavity

seem to play an important role in gastric NO generation (81). Webb *et al.* showed in a study that ingestion of beetroot juice, which contains large amounts of nitrate, caused a lowering of blood pressure (82).

Soluble urokinase plasminogen activator receptor (suPAR) is the soluble form of urokinase-type plasminogen activator receptor (uPAR) and was identified in 1991 (83). uPAR is present at different types of immune cells including activated T-lymphocytes and macrophages. SuPAR is found in blood, plasma, serum, urine and cerebrospinal fluid. It reflects an overall activation of unspecific systemic inflammation and has shown to be up-regulated in human immunodeficiency virus (HIV), malaria and sepsis (84-86). Moreover, suPAR levels have shown to be associated with increased risk of respiratory cancer (87). Previous studies have shown suPAR to strongly correlate with TNF and also with CRP (88, 89). SuPAR blood concentration has also shown to be a risk marker in the general population (90). Whether suPAR itself promotes pro-inflammatory response, or if it is a result of increased immune activation is yet to explore.

Ghrelin is a peptide hormone, playing an important role in appetite regulation and energy metabolism (91). It has also shown to be involved in immune responses and inflammatory processes (92, 93). Serum ghrelin levels have been reported to be significantly higher in CD and UC patients with active disease compared with patients in remission or healthy controls (94, 95). Furthermore, serum ghrelin levels have shown to correlate with CRP in IBD patients (95). Sung *et al.* found plasma ghrelin to increase following infliximab infusion (96).

As the name suggests, endothelin-1 is produced in vascular endothelial cells during physiological conditions, promoting vasoconstriction (97). Moreover, mono- and polymorphonuclear leucocytes in mucosa can produce endothelin. Boros *et al.* showed in a rat model that endothelin-1 causes leukocytes to slow down and adhere to the endothelium (98). Cui *et al.* found endothelin-1 to be a strong chemoattractant for human neutrophils and monocytes (99). A link between leukocyte chemoattraction, mast cell breakdown and endothelin-1 has been suggested in another study by Boros *et al.* (100). Murch *et al.* found increased endothelin-1 levels in intestinal tissue from CD and UC patients (101). This was confirmed by Kanazawa *et al.* who reported high plasma levels of endothelin in both CD and UC (102).

1.5.2 Fecal calprotectin

Fecal calprotectin is a sensitive marker for IBD and can separate IBD from irritable bowel syndrome (IBS) (103). However, calprotectin is not disease-specific and may rise in conditions such as colon cancer and non-steroidal anti-inflammatory drug (NSAID)-induced inflammation (104-107). Calprotectin is stable in room temperature and resistant to proteolysis, which makes it a feasible marker in clinical routine (67). Strong correlations between calprotectin and histological and endoscopic findings have been confirmed in several studies (108-111).

1.5.3 Luminal nitric oxide

Furchgott and Zawadzki described endothelium-derived relaxing factor (EDRF) in 1980 (112). EDRF was later found to be NO (113, 114), which was awarded the Nobel prize in 1998. NO is involved in numerous mechanisms throughout the body. First of all, it is a potent vasodilator, but has also important functions in e.g. platelet aggregation, mucus generation and leukocyte adhesion to the endothelium (115-118). Through its tiny size and lipophilic quality, NO can diffuse freely across membranes. There are different NO-formation pathways in the body: the enzymatic and the enzyme-independent way. The former pathway is oxygen dependent; amino acid L-arginine reacts with oxygen via NOS, deriving NO and L-citrulline (119). During inflammation, the inducible NOS (iNOS, NOS2) produces high amounts of NO. The latter pathway acts as an NO buffer during hypoxia. Organic nitrite and nitrate can via chemical reduction generate NO also under conditions with low oxygen availability. Depending on duration and anatomic site for the NO production, the effect can be both anti-inflammatory and cytotoxic. Nitrite-derived NO stimulates mucosal blood flow and mucus production and decreases leakage across the epithelium, hence plays a significant role in host defence (120, 121). Herulf *et al.* showed an increase of luminal NO in IBD patients, which was later confirmed also in infective gastroenteritis, coeliac disease and collagenous colitis (122-125).

2 AIMS OF THE PRESENT THESIS

The general aim of this thesis was to study markers of inflammation during pharmacological therapy of inflammatory bowel disease and acute colitis.

Specific aims:

- To study the long-term outcome concerning blood status and systemic inflammatory parameters, clinical activity index and quality of life in patients treated with infliximab
- To evaluate the short- and long-term outcome in systemic inflammatory markers along with clinical activity in patients undergoing the first year of scheduled infliximab therapy
- To study the temporary levels of fecal calprotectin and plasma-CRP along with clinical activity and clinical outcome during infliximab induction therapy
- To evaluate luminal nitric oxide and plasma-CRP levels in association with response and colectomy rate in patients with acute colitis treated with glucocorticosteroids.

3 MATERIAL & METHODS, STUDY I-IV

3.1 STUDY SUBJECTS

Permission to perform the studies was obtained from the regional ethics committee and all subjects gave their informed consent prior to entering the study. Diagnosis of the patients with IBD (154 with CD and 8 with UC) included in paper I-III was based on clinical, endoscopic and histological criteria (Table V). The patients in paper IV ($n = 45$) were diagnosed with acute colitis based on clinical and endoscopic criteria. Paper II includes ten healthy volunteers.

Table V. Patients included in the thesis

| | Paper I | Paper II | Paper III | Paper IV |
|--------------------------|---------|----------|-----------|----------|
| CD | 103 | 22 | 29 | N/A |
| Colon | 75 | 15 | 20 | - |
| Ileo-colonic | 4 | 5 | 8 | - |
| Small-intestine | 24 | 1 | - | - |
| Colon and upper GI-tract | - | 1 | 1 | - |
| Fistulae | 24 | N/A | 8 | N/A |
| UC | - | - | 8 | N/A |
| Left-sided | - | - | 2 | - |
| Extensive | - | - | 6 | - |
| Acute colitis | - | - | - | 45 |

Abbreviations: CD = Crohn's disease, UC = ulcerative colitis, GI = gastrointestinal, N/A = Not applicable. The numbers represent number of individuals.

3.2 LABORATORY METHODS

3.2.1 Blood samples

Blood samples were drawn and centrifuged at 4°C for 10 minutes at 3000 rpm. Plasma and serum was collected and stored at -70°C until analysis. All analyses were carried out in duplicate and according to the manufacturers' instructions.

3.2.2 Sampling of rectal NO

The silicone Argyle™ catheter (Sherwood Medical, Tullamore, Ireland) was used to sample rectal NO (paper IV). Using lubrication gel, the catheter tip was positioned 10 cm above the anal sphincter. Ten mL of ambient air in a syringe was used to inflate the balloon. The air was left for 10 minutes to equilibrate with the luminal gas (126). Thereafter, the gas was withdrawn from the balloon, diluted to a final volume of 50 mL and analyzed with correction for dilution. Analyses were made within 10 minutes of sampling.

3.2.3 Chemiluminescence

Nitrite (paper I) was analyzed by reductive cleavage and subsequent determination of NO released into the gas phase by chemiluminescence. Plasma samples were introduced into a reducing solution (45 mmol potassium iodide and 10 mmol iodine per litre in acetic acid) via a gas-tight syringe. The reduction chamber was kept at 60°C controlled by a flow of warm water. The reducing solution was constantly bubbled with nitrogen, used as carrier gas for NO. An acid trap (sodium hydroxide 1M, 0° C) caught traces of acid before the NO gas was analyzed in the chemiluminescence system (Aerocrine AB, Stockholm, Sweden) (127).

Measurements of luminal NO (paper IV) were made employing a CLD 700 (EcoPhysics, Dürnten, Switzerland). In this instrument, gaseous NO reacts with an excess of ozone (O₃) to form NO₂, which when it turns from its excited state (NO₂*) to its ground state releases excess energy as photons and emitted light. The light can be quantified via a photo-sensitive surface and amplified in a photomultiplier. The light production is directly proportional to the amount of NO, linear within the interval 1-100 000 parts per billion (ppb).

3.2.4 Determination of IgA and IgG-ASCA

ASCA (paper I) was analyzed with an in-house method. Fifty gram of *Saccharomyces cerevisiae*, baker's yeast (Svenska Jästfabriken AB, Rotebro, Sweden), was heat-killed, pooled at a concentration of 10⁸ particles/mL and stored at -20° C. After thawing, the yeast particles were resolved in 1 mL 0.9% NaCl, counted in a Bürker chamber and re-suspended to a final concentration of

10^6 particles/mL. Ten μ L human serum albumin (200 mg/mL, Pharmacia, Uppsala, Sweden) was added per mL yeast particles to avoid unspecific binding. Serum from healthy blood donors (group AB+) was used as background control.

Sixty μ L of the *Saccharomyces cerevisiae* solution was mixed with 20 μ L serum (patient or control) and incubated in room temperature for 30 min. Antibody-antigen reaction was completed by adding 100 μ L of secondary antibody fluorescein isothiocyanate (FITC)-labelled rabbit anti-human IgG and IgA (DAKO A/S, Glostrup, Denmark) in 4° C phosphate buffer, incubated for 30 min, washed and centrifuged. After decanting, the pellets were dissolved in 0.5 mL phosphate buffer and analyzed in an EPICS XL-MCL flow-cytometer (Beckman Coulter Inc., Fullerton, CA, USA).

3.2.5 Near infrared particle immunoassay

High-sensitive CRP (paper II) was analyzed with high-sensitive near infrared immunoassay rate (NIPA rate) and turbidimetry by a UniCel DxC 800 (Beckman Coulter Inc., Fullerton, CA, USA). The principle is that an anti-CRP antibody-coated particle binds to CRP in the presence of patient sample, forming an insoluble aggregate. This non-soluble aggregate makes the solution murky, resulting in an increased turbidity. The absorbance is measured at 940 nm and is proportional to the concentration of CRP (interval 0.2-380 mg/L).

3.2.6 Enzyme-linked immunosorbent assay

Serum calprotectin and suPAR (paper II) were analyzed with enzyme-linked immunosorbent assay (ELISA) (PhiCal® MRP 8/14 ELISA, Immundiagnostik, Bensheim, Germany, and suPARnostic® Standard Kit, Virogates, Birkerød, Denmark). This assay utilizes a “sandwich” technique with two monoclonal antibodies binding to the agent. The microplate is covered with antibodies which during the first incubation step bind to the added sample agent. In the next incubation step, a second antibody is added followed by a chromogenic substrate. During the last step, colour is developing proportional to the amount of agent in the sample. The reaction is terminated by adding acid to the wells and the absorbance is measured in a plate reader (NanoQuant infinite200, Tecan, Tecan Austria GmbH, Salzburg, Austria). The absorbance for both calprotectin and suPAR was read at 450 nm against 620 and 650 nm respectively, as reference.

3.2.7 Radioimmunoassay

Plasma ghrelin and endothelin (paper II) were analyzed with radioimmunoassay (RIA) (128). Ghrelin was analyzed with Ghrelin Total RIA Kit (Linco Research, St Charles, MO, USA) and endothelin by Euria-Endothelin, RB 304 (Euro-Diagnostica AB Malmö, Sweden). In RIA, a fixed concentration of labelled tracer antigen is incubated with a constant dilution of antiserum. When unlabelled antigen is added to the system, there is competition between labelled tracer and unlabeled antigen for the number of binding sites on the antibody. Both methods utilize ^{125}I to label the tracer. The amount of tracer bound to antibody will decrease as the amount of unlabelled antigen increases.

3.2.8 Griess reaction

Total nitrite in serum (paper II) was analyzed with the Griess reaction (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA) (129). This analysis is carried out in a two-step process. In the first step, nitrate (NO_3^-) is converted to nitrite (NO_2^-) utilizing nitrate reductase. In the second step, Griess-reagents are added to form a purple azo-compound. The colour is proportional to the amount of nitrite in the sample and measurement of the absorbance at 540 nm is used to determine the total nitrite concentration (NanoQuant infinite200, Tecan, Tecan Austria GmbH, Salzburg, Austria).

3.3 STATISTICS

GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) was used for statistical analyses (paper I-IV). In paper II, statistical analyses were also carried out with Stata 11.0 (StataCorp LP, College Station, TX, USA). Values are presented as mean \pm SEM when data showed normal distribution. If data did not pass normal distribution test (employing Kolmogorov-Smirnov test), they are presented as median and interquartile range (IQR). $P < 0.05$ was considered statistically significant.

In paper I, the evaluation of test results before and after infliximab infusion was carried out with Student's t-test or Wilcoxon matched-pair test. Trends over time

were analyzed with unmatched Student's t-test employing Welch's correction. Correlation was analyzed by linear regression.

In paper II, results were calculated by Wilcoxon test for paired groups and Mann-Whitney test for unpaired data. Correlations were tested utilizing Spearman nonparametric correlation.

In paper III and IV, sensitivity and specificity were analyzed by receiver operating characteristic (ROC) curve. Survival curves were analyzed with Kaplan-Meier analysis along with log-rank test. Spearman nonparametric test served for correlation analyses.

4 RESULTS

4.1 PAPER I

A total of 103 CD patients were monitored and were given altogether 1061 infusions. Seventy-nine patients had inflammation located in the ileo-cecal or colonic mucosa, 24 patients had fistulizing disease. Of those, 92 patients (89%) responded to infliximab therapy and received a median of seven infusions during a period of up to five years. Infliximab was withdrawn in the 11 non-responders. In the initial responding group, 39 patients (42%) terminated infliximab therapy due to loss of response (n=11), side-effects (n=12) or other reasons (n=16). Mild circulatory side-effects could in most cases be handled by slowing the infusion rate or by treatment with corticosteroids or antihistamine. However, 12 patients experienced side-effects of such severe nature that infliximab had to be withdrawn.

4.1.1 Clinical response and quality of life

Clinical response according to the HBi decreased over the first year from 7.7 to 5.5 ($p<0.0001$) and remained steady over the following four years.

The four dimension-based visual analogue scale Short Health Scale (SHS) showed that quality of life improved significantly in each dimension with infliximab infusions, most markedly in the symptom burden-related question.

4.1.2 Blood chemistry

Hemoglobin and albumin increased with infliximab infusions, and platelets and leukocytes improved over time. CRP decreased with treatment and remained low the following years. There were no signs of renal affection evaluated as creatinine levels.

Nitrite and ASCA were analyzed in subgroups. Nitrite levels tended to increase after infliximab infusions, from 74.8-125.3 nmol/L ($p<0.001$) over the first year. No relationships were found between ASCA and infliximab therapy.

4.1.3 Correlations

A weak but significant correlation was found between HBi and CRP delta values ($r=0.27$, $p<0.001$). No correlations were found between HBi and other blood chemistry components.

4.1.4 Summary and conclusions

The enhanced blood chemistry values mirrors an overall improvement in patients responding to infliximab. The acute inflammation was rapidly suppressed as shown by CRP. Hemoglobin, albumin, platelets and leukocyte levels were stabilized over time, along with positive results in clinical activity index and quality of life. These results confirm infliximab to be effective in both short- and long-term outcome in patients with moderate to severe CD.

Stenosis and fistula development are common in the progressive course of CD, and an HBi normalization (<5 points) should be a hard goal in the evaluation of infliximab therapy outcome. A prompt symptomatic relief was shown already within one week.

In the search of a correlation between clinical activity and biomarkers, we found CRP to be the most reliable one, however only weakly associated with patients' symptoms evaluated as HBi.

4.2 PAPER II

Paper II included 22 CD patients undergoing the first year of scheduled infliximab therapy. Fifteen patients had disease located to the colon, five patients in the ileum and colon, one patient in colon and upper gastrointestinal tract and one patient in ileum only. Seven patients were anti-TNF-naïve at the study start and 15 patients had received a maximum of five previous infusions. Blood samples were drawn prior to infliximab infusion and at follow-up visit one week later. This routine was repeated after six months (three to four infusions later). Ten healthy volunteers served as controls.

4.2.1 Clinical activity

Out of 22 paired observations, 15 (68%) had a clinical response or reached clinical remission at the first post-infusion visit. HBi decreased from 8.3 (5.0-11.3) to 4.5 (3.4-7.3) ($p<0.01$). At the second pre-infusion visit, HBi levels had returned to baseline levels, but improved in all patients but one at the second post-infusion visit, from 7.0 (4.0-13.1) to 5.3 (2.9-7.8) ($p<0.001$).

4.2.2 Blood chemistry markers

Hs-CRP showed a great variability in concentrations and was separated from healthy controls solely at the 6-month pre-infusion visit. It did however decrease with infliximab, but significantly only at the second post-infusion visit.

Calprotectin in serum decreased with infliximab infusions at both follow-up occasions but did not reach normal levels, hence were separated from healthy controls at all four study visits. Total nitrite tended to increase with infliximab infusions and was separated from healthy controls throughout the study. SuPAR levels did not change with infliximab infusions, but instead indicated a constant activation of the inflammatory response being elevated at each study visit as compared with controls. Ghrelin decreased with infliximab infusions (significant at the first post-infusion visit), but did not differ from controls at any study visit. Endothelin decreased with infusions, significantly at the 6-month follow-up. The pre-infusion levels were similar to those of controls, but lower after infusions.

4.2.3 Correlations

A correlation was found between HBi and hs-CRP (Spearman $r=0.32$, $p<0.05$). None of the other markers correlated with activity index. However, hs-CRP correlated with serum calprotectin (Spearman $r=0.35$, $p<0.05$) as well as with suPAR (Spearman $r=0.33$, $p<0.05$).

4.2.4 Summary and conclusions

Serological inflammatory markers were studied as an attempt to monitor disease activity in a minimal invasive manner. The correlation between HBi and hs-CRP

favours this marker for evaluation of anti-TNF therapy response. However, the great variability in hs-CRP concentrations as well as its non-IBD specificity makes it a less useful marker.

Total nitrite represents total nitrate and nitrite levels. Nitrate is present in the blood stream in far higher amounts than nitrite and both compounds contribute to NO generation. Nitrite-derived NO is suggested to act cytoprotectively to myocardial ischemia (130). Attenuated ischemic conditions and hence increased oxygen availability might theoretically be contributing to the increased nitrite concentrations in IBD patients following infliximab infusion. However, it does not explain the difference from healthy controls.

Infliximab seems to decrease inflammatory activity promptly with obvious improvement one week after infusion. However, it does not normalize inflammatory markers over long-term scheduled therapy, illustrated by the 6-month infusion visit concentrations which were similar to baseline levels. Also, the constantly increased levels of calprotectin, nitrite and suPAR compared with healthy controls indicate an ongoing subclinical inflammatory activity.

There were no differences in inflammatory markers or activity index between anti-TNF naïve patients and those who had received previous infusions prior to study start.

4.3 PAPER III

Fecal calprotectin and CRP along with clinical activity were evaluated in 37 IBD patients (29 with CD and 8 with UC) during infliximab induction therapy (infusions week 0, 2 and 6). Of the CD patients, 20 had colonic disease, 8 had ileocolonic disease and one had disease located in the colon and stomach. Eight patients had concurrent fistulizing disease. Of the UC patients, six had extensive colitis and two had left-sided colitis. Twenty hospitalized patients received infliximab due to acute exacerbation and 17 as elective out-patient induction therapy. At baseline, six patients had mild to moderate endoscopic inflammation and 31 moderate to severe. Eleven patients had azathioprine and corticosteroids, four had azathioprine monotherapy, 14 patients had corticosteroid monotherapy and eight had no IBD-related pharmacological treatment.

4.3.1 Clinical activity

Based on the HBi and partial Mayo score, 31 patients (84%) responded to infliximab and 24 (65%) reached clinical remission after induction therapy. Six patients (3 CD, 3 UC) did not respond.

4.3.2 Fecal calprotectin and CRP

At baseline, the median fecal calprotectin level in the total group was 2060 (1495-5507) $\mu\text{g/g}$ and CRP 13 (6.5-35.5) mg/L . In the responding group median calprotectin was 210 (70-358) $\mu\text{g/g}$ after induction therapy compared with non-responders with a median value of 1405 (304-2171) $\mu\text{g/g}$. The CRP-levels in the same groups were 2 (1-4) and 5.5 (3.5-27.5) mg/L , respectively.

4.3.3 Predictive values

The fecal calprotectin ROC analysis best cut-off point was 221 $\mu\text{g/g}$ (sensitivity 80%, specificity 67%), with an area under the curve computed to 0.81. Kaplan-Meier analysis of responders showed that an FC level exceeding 221 $\mu\text{g/g}$ after induction therapy was associated with an incident (need for surgery, shortening of infusion interval from every eight to every six weeks, dosage increase from 5 mg/kg to 10 mg/kg bodyweight) within 24 weeks after induction therapy ($p < 0.05$). In patients with calprotectin $< 221 \mu\text{g/g}$, two out of 16 (13%) had an incident within 24 weeks. Among those with calprotectin $> 221 \mu\text{g/g}$ the corresponding number was 8 out of 15 (53%) within the same period.

The CRP ROC analysis best cut-off point was 2.5 mg/L (sensitivity 70%, specificity 76%, area under the curve 0.78). The Kaplan-Meier curve showed that a $\text{CRP} > 2.5 \text{ mg/L}$ after induction therapy was associated with an incident within 24 weeks ($p < 0.05$). In patients with CRP levels below 2.5 mg/L , three out of 19 (16%) had an incident within 24 weeks. In patients with CRP levels exceeding 2.5 mg/L the corresponding number was 7 out of 12 (58%).

4.3.4 Concomitant azathioprine treatment

When comparing patients who were concomitantly treated with azathioprine with those who were not, we found significantly higher CRP levels after induction therapy in patients without azathioprine with median values of 4 (2-7.5) mg/L compared to 1 (1-3) mg/L in patients with combined therapy ($p<0.05$). None of the non-responders were concomitantly treated with azathioprine.

4.3.5 Correlations

When combining pre- and post-infusion data, clinical disease activity correlated with fecal calprotectin (Spearman $r=0.62$, $p<0.0001$) as well as with CRP (Spearman $r=0.60$, $p<0.0001$). Furthermore, calprotectin correlated with CRP (Spearman $r=0.59$, $p<0.0001$).

4.3.6 Summary and conclusions

The study shows that clinical indices correlate well with fecal calprotectin and CRP during infliximab induction therapy at week 0, 2 and 6, and furthermore, that a calprotectin level exceeding 221 $\mu\text{g/g}$ after induction therapy is associated with an incident within the following 24 weeks. In patients responding to infliximab, the clinical indices, fecal calprotectin and CRP decreased significantly from baseline at 12 weeks.

In this study, CRP showed no capability in predicting flare-up. The best cut-off point (2.5 mg/L) lies within the normal interval (<3 mg/L). CRP levels remained slightly increased in patients without concomitant azathioprine therapy. A similar result has been reported by Sokol *et al* (131). None of the non-responders were concomitantly treated with azathioprine. These results support the notion that combination therapy of azathioprine and infliximab is superior to monotherapy (39).

Only four patients had calprotectin levels below 50 $\mu\text{g/g}$ after induction therapy, even though 25 reached clinical remission. Patients responding to treatment with sustained elevated fecal calprotectin levels should be closely monitored since

there is an increased risk of flare-up. Patients in clinical remission along with calprotectin levels <221 µg/g are at low risk.

4.4 PAPER IV

Luminal nitric oxide was analyzed in 45 patients hospitalized due to acute colitis and treated with corticosteroids as first-line therapy to subdue the inflammation. NO was measured before GCS onset and on the third day of treatment. Some of the patients had established IBD diagnoses (CD or UC) whereas others were naïve with no specific diagnosis other than acute colitis. CRP was analyzed as a comparative marker for acute inflammation.

4.4.1 Clinical activity

Response to therapy was evaluated as a decrease of ≥ 3 points in disease activity index (DAI, UC) or HBi (CD). In patients with no yet established sub-diagnosis, the HBi was used.

Baseline activity index in the total group was 10 (8-11), which decreased to 7 (4.5-9.5) on day 3 after GCS onset. When grouping patients due to surgical outcome, activity index over the first 3 days decreased significantly in patients spared from colectomy, from 10 (8-11) to 7 (4-8.5) ($p < 0.001$). The corresponding values in colectomized patients did not differ over time [11.5 (9-12) to 11 (7.5-13.5)].

4.4.2 NO and CRP

In patients responding to GCS ($n = 17$, 38%), baseline median NO level was 14444 (3200-24074) ppb and did not change significantly at day 3 [11833 (1306-30950) ppb]. In the non-responding group of patients, the corresponding levels were 1675 (313-5109) ppb and 1111 (483-3996) ppb, respectively. The non-responders had significantly lower NO-levels at both baseline and day 3 ($p < 0.05$). Out of 28 non-responders, seven received azathioprine, seven started infliximab therapy and 14 underwent colectomy.

When comparing NO-levels in patients who were colectomized (n=14) within one month from hospitalization with those who were not (n=31), significantly lower levels were found in colectomized patients at both baseline and day 3 compared with responders ($p<0.01$). In patients spared from colectomy, baseline NO was 8450 (3167-15111) ppb, while in colectomized patients the level was 450 (220-1663) ppb. The day 3 levels were 4800 (750-24400) ppb and 590 (300-1583), respectively. The levels did not change over time within the groups.

Baseline CRP in the total group was 33 (7-41.5) mg/L. Responders and non-responders did not differ in baseline levels; 29.5 (12-38.5) and 33 (7-52) mg/L, respectively. Colectomized patients had higher baseline CRP levels than those who eluded surgery; 40 (27-48) and 22 (7-38.5) mg/L respectively ($p<0.05$).

4.4.3 Predictive values

The best NO cut-off point to differentiate between GCS responders and non-responders was 4772 ppb, with a sensitivity and specificity of 71% and 75%, respectively.

When performing ROC-analysis on luminal NO as a predictor for colectomy, the best cut-off point was 2239 ppb, with a sensitivity and specificity of 86% and 81%, respectively, and an area under the curve of 0.88. Kaplan-Meier analysis showed that a baseline NO level below 2239 ppb was significantly associated with colectomy within one month ($p<0.0001$). Twelve out of 18 (67%) in patients with baseline NO <2239 ppb were colectomized. The corresponding number in patients with baseline NO >2239 ppb was two out of 27 (7%).

Studying the predictive value of CRP, ROC-analysis was not significant.

4.4.4 Summary and conclusions

This study shows that luminal nitric oxide does not change over the first three days of GCS therapy; hence, GCS does not seem to have an immediate effect on NO production.

Patients with increased baseline NO levels seem to benefit from GCS therapy in a higher extent than do patients with lower baseline values, in response as well as

in colectomy rate. The low values in non-responders and colectomized patients may reflect an extended destruction of NO-producing mucosa due to severe inflammation.

The clinical activity indices showed that 3 days seem to be enough to evaluate therapy response or failure. In patients with a poor response to GCS along with low luminal NO, rescue therapy measures are indicated.

5 GENERAL DISCUSSION

To be diagnosed with a lifelong disease such as IBD may naturally result in worry and questions regarding disease progression, therapy options and outcome. Seeing that disease symptoms and severity vary widely within this patient group and flares most of the times are not possible to predict, many of these questions will be left unanswered (132). Available pharmacological therapy is symptom alleviating, not curing. Many patients will develop steroid dependency or resistance over years, requiring therapy supplement according to the step-up regimen. Activity indices convey essential disease course information, but need to be complemented with objective inflammatory markers to evaluate the true inflammatory activity. Both systemic and local markers of inflammation contribute with important information of the situation. Systemic CRP has shown to correlate strongly with disease activity index in CD, whereas local fecal calprotectin correlates robustly with endoscopic indices.

In this thesis, inflammatory markers have been studied at systemic level (paper I - IV) as well as locally in the gut (paper III and IV). The study shows that infliximab effectively ameliorates the IBD symptoms and that clinical response and quality of life improves within one week from infusion. Also, blood chemistry markers improve during this period. However, inflammatory markers did not constantly follow the same trend, most clearly illustrated by calprotectin, nitrite and suPAR. Moreover, rectal NO did not change over the three-day follow-up during GCS therapy. Fecal calprotectin can be used as a predictor of flare-up during infliximab therapy, whereas luminal NO showed strong colectomy predicting capability during GCS therapy.

In paper I, improvements in hemoglobin, albumin and CRP over short- and long-term infliximab therapy were confirmed. In IBD patients, chronic inflammation or intestinal blood loss, together with malfunctioning duodenal iron absorption, cause a negative iron balance and anemia. One third of the IBD population has hemoglobin concentration below 120 g/L (the lower reference limits are 120 g/L in women and 130 g/L in men) (133). Hemoglobin is regularly monitored in IBD patients to assess the presence or absence of anemia, which it is the most common systemic complication in IBD and has a great impact of quality of life (134, 135). Serum albumin concentration has been shown to correlate with CRP-

levels in UC patients (136). The improved circulating albumin levels following infliximab infusion reflect a decreased leakage of circulating albumin to intercellular space, i.e. less inflammation. The decrease in CRP-levels illustrates a prompt inhibition of the acute inflammation. CRP is still used as gold standard marker in evaluation of systemic inflammatory activity. It is cheap and rapid to analyse, but lacks disease specificity, making it unspecific to rely on solely. Albeit, CRP was the only marker correlating with activity index in paper I and II. This is in line with findings from other groups (57, 137).

Inflammatory markers improved with infliximab infusions in paper II. However, the expected normalization over short- (one week) and long-term (six months) was absent and furthermore, markers tended to return to baseline level at the second pre-infusion visit. These results indicate either an insufficient time-to-follow-up, or merely a persistent activation of inflammatory response unaffected by infliximab. This was illustrated especially by suPAR, calprotectin and nitrite being constantly separated from healthy controls. Paper II describes suPAR levels for the first time in inflammatory bowel disease. SuPAR has no diagnostic value, but has shown prognostic strength in several infectious diseases (85, 138, 139). SuPAR levels in CD patients were as expected considerably lower than for example in sepsis studies, but still significantly increased compared with healthy individuals. Moreover, suPAR did not change with infliximab infusions. The course of disease does not seem to change with infliximab therapy, managing only to decrease the short-term inflammation. Moreover, patients' naïve to anti-TNF therapy at study start showed no difference in outcome from previously treated patients after sub-analysis. A possible explanation might be that all anti-TNF naïve patients were treated with other anti-inflammatory drugs (GCS, azathioprine) prior to infliximab onset, which probably already suppressed the inflammatory activity. The great variability in hs-CRP levels in paper II could be explained by the fact that a majority of the patients were, according to the step-up strategy, treated with GCS and/or immunosuppressants prior to infliximab onset. Neither endothelin nor ghrelin were suitable as markers of inflammation in this study. Ghrelin levels did not differ between patients and controls at any study visit, even though it tended to decrease with infliximab infusions. Ates *et al.* found higher serum ghrelin levels in IBD patients with active disease than patients in remission, but differences were not significant between the total group

of IBD patients and healthy controls (95). Thus, ghrelin may play a role in inflammation, but does not seem useful as marker of infliximab therapy evaluation. Endothelin-1 has been suggested to be involved in IBD inflammation (101). Furthermore, the non-selective endothelin-receptor antagonist bosentan has shown to ameliorate inflammation in animal models (140, 141). However, since a clear role of endothelin in IBD has not been confirmed, all forms of endothelin; endothelin-1, -2 and -3 were analyzed. Pre-infusion levels were similar to healthy control levels, whereas post-infusion levels were significantly lower. The results cannot be explained from an inflammatory perspective, but rather from general aspects such as age, physical activity or prandial status.

A nitrite and total nitrite increase tendency with infliximab infusions was shown in two publications (paper I and II), employing two separate analysis methods. This favours the suggestion of nitrite and hence NO acting anti-inflammatory (142, 143). During inflammation, superoxide destroys NO to form peroxynitrite which can isomerize to form nitrate. With less superoxide and hence less nitrate derived, NO might instead form nitrite. Moreover, TNF up-regulates the endothelial arginase expression, which depletes NOS of L-arginine, leading to a decrease in NO production (144). Anti-TNF therapy might therefore give the opposite result, with increased NO and hence nitrite levels. An additional hypothesis is that the patients improve their plasma nitrite levels because of a richer dietary uptake of nitrite and nitrate once the inflammation is alleviated.

Paper II and III showed that serum and fecal calprotectin do not decrease to levels of those in healthy controls with infliximab therapy, irrespectively of systemic or local analysis. Calprotectin was described in 1980 and has rapidly been implemented as a fecal marker of inflammation in clinical routine, as it closely reflects the granulocyte migration through the gut wall and hence inflammatory activity (145). In paper III, only four patients had levels below the reference limit after induction therapy even though 25 patients reached clinical remission according to disease activity indices. Evidently, clinical activity indices underestimate the inflammatory activity often found in patients with quiescent disease (146).

In paper III, fecal calprotectin was found to decrease significantly in responders following infliximab induction therapy and moreover, to be a predictor of

incidents as signs of flare-up the following 24 weeks. This is in line with previous findings by Garcia-Sanchez *et al.* who found that CD and UC patients with calprotectin exceeding 200 respectively 120 $\mu\text{g/g}$ relapsed 4 respectively 6 times more often than patients with lower values (71). Gisbert *et al.* found a significant risk for relapse at a cut-off level of $>150 \mu\text{g/g}$ in IBD patients in stable remission (147). Similar findings have also been reported in pediatric IBD populations (148).

In paper IV, NO levels in acute colitis patients undergoing GCS therapy were shown to differ between responders and non-responders. However, NO levels did not decrease over the first three days of therapy, indicating that NO seems either not to be affected by GCS, or the change is delayed and occurs after a prolonged period (149). Since NO levels remain at baseline level after three days of therapy, baseline level is sufficient to predict outcome. Patients with increased NO seem to benefit more from GCS therapy. It is not obvious why patients with higher NO levels (reflecting increased inflammatory activity) would respond better to GCS. The results can be compared to those from Louis *et al.*, who found patients with $\text{CRP} > 5 \text{ mg/L}$ to respond better to therapy than those with lower levels (58). In paper III, patients with the most severe endoscopic inflammation had lower fecal calprotectin values than patients with moderate endoscopic inflammation, possibly caused by an increased mucosal secretion. This theory might also be applied in rectal NO and acute colitis, where a damaged epithelium could result in reduced NO production. In the same way as fecal calprotectin, luminal NO measures inflammation close to the inflamed area and should thus give a better estimate of the inflammatory activity. Lundberg *et al.* showed in 1994 that NO values were higher in samples obtained close to lesions than farther away from inflamed area in UC (150).

In all four studies, only weak correlations between clinical activity index and inflammatory markers were found. Not surprisingly, the strongest correlation was found between fecal calprotectin and clinical activity index (paper III). Fecal calprotectin has an advantage of measuring inflammation on site. Elevated CRP levels are strongly correlated with CD, whereas in UC, CRP increase is only moderate or absent (151). This might have contributed to the variation in CRP concentrations in paper III and IV, since both CD and UC patients were included.

The activity indices' underestimation of the actual inflammatory activity may not be of importance in evaluating therapy outcome since it for example efficiently separates responders from non-responders. However, biological therapy is very expensive, and when the patient has reached stable clinical remission, the question of anti-TNF-withdrawal might be raised. One must on that occasion bear in mind that a subclinical inflammation most likely is present and hence, withdrawal might elicit a rapid flare-up, with less chance of regained anti-TNF response due to development of antibodies to infliximab (152, 153).

From the patients' point of view, increased wellbeing, quality of life and decreased symptom burden are probably the primary goals. However, due to the risks brought by long-term continuous inflammation, it is of most importance to eliminate as much as possible of inflammatory activity. A combination of clinical activity index and eligible inflammatory markers, chiefly circulating CRP, calprotectin, nitrite and suPAR, fecal calprotectin and luminal NO, can form strong tools in clinical practise and evaluation of medical therapy.

6 CONCLUSIONS

Biological therapy with infliximab brings rapid improvement in clinical activity, quality of life and blood chemistry. The improvement maintains over years.

Systemic calprotectin, nitrite and suPAR are valuable markers in evaluation of inflammatory activity during the first year of infliximab therapy, whereas ghrelin and endothelin showed weaker results. SuPAR was shown for the first time in inflammatory bowel disease to be constantly elevated in CD patients treated with infliximab, compared with healthy controls.

Clinical activity index correlates with fecal calprotectin and CRP during infliximab induction therapy. Fecal calprotectin can be used as a predictor of flare-up following infliximab induction therapy, being superior to CRP in this aspect. In mild to moderate IBD, fecal calprotectin correlates with baseline endoscopic index. In more severe disease, this correlation is lost. Infliximab therapy combined with azathioprine seems beneficial as evaluated by CRP.

Baseline luminal nitric oxide can be used as a predictor of colectomy in acute colitis patients treated with GCS. Patients with low baseline NO and poor response to GCS should be given rescue therapy in order to prevent colectomy. Patients who underwent colectomy had higher baseline CRP and lower NO concentration compared with patients spared from surgery.

Among the systemic inflammatory markers, hs-CRP showed the most useful properties due to its association with clinical activity index. Of the local inflammatory markers, fecal calprotectin and luminal NO were very useful in terms of relapse and surgery.

7 SUMMARY IN SWEDISH

Inflammatorisk tarmsjukdom är ett samlingsnamn för diagnoserna Crohn's sjukdom och ulcerös colit. Dessa sjukdomar är kroniskt återkommande med skov som ger svåra symtom (blod- eller slemblandade diarréer, smärta, viktnedgång). Crohn's sjukdom kan drabba hela mag-tarmkanalen från mun till anus och kan även medföra fistelproblematik och förträngningar i tarmen. Ulcerös colit drabbar tjocktarmen med kontinuerlig inflammation. Båda patientgrupperna löper en avsevärt högre risk än normalbefolkningen att drabbas av colorektalcancer. Tillgänglig behandling syftar till att inducera och bevara symtomfrihet. Tillförlitliga markörer skulle kunna underlätta utvärdering av behandlingssvar och inflammatorisk aktivitet på ett objektivet sätt. Vi ville med det här projektet studera olika markörer i blod, avföring samt rektalgas vid samtidig behandling av inflammatorisk tarmsjukdom för att se hur de förändras under behandling samt om de stämmer överens med de index som används för att uppskatta sjukdomsaktivitet.

I det första arbetet följde vi Crohn-patienter före och efter infliximabinfusion under första behandlingsåret och därefter årligen i upp till fem år. Vi visade att ämnen i blod som påverkas av inflammation normaliserades med behandling och att behandlingseffekten kvarstod över lång tid. Patienterna rapporterade en ökad livskvalitet i och med behandling.

I arbete två analyserade vi sex inflammationsmarkörer i plasma och serum före och efter infliximabinfusion hos Crohn-patienter under deras första behandlingsår med infliximab. Som jämförelse analyserade vi även samma markörer hos friska kontroller. Resultaten visade att inflammationen minskade med behandling, men flera av markörerna var fortfarande konstant förhöjda jämfört med friska kontroller, vilket indikerar en ständigt pågående subklinisk inflammation.

I arbete tre följde vi fekalt calprotectin och plasma-CRP hos patienter med Crohn's sjukdom och ulcerös colit under induktionsbehandling med infliximab. Hos patienter som svarade på behandling sjönk calprotectin och CRP samtidigt som aktivitetsindex förbättrades. Calprotectin kunde även användas för att prediktera nytt skov inom följande 24 veckor.

I det fjärde arbetet mätte vi NO i rektalgas före och tre dagar efter påbörjad kortikosteroidbehandling hos patienter som sjukhusvårdades för skov av akut colit. Lyckas man inte häva inflammationen i tid kan patienten behöva genomgå colektomi (tjocktarmen opereras bort). De patienter som svarade på behandling hade vid bägge mättillfällena högre NO-värden än de som inte svarade. NO kunde även användas för att prediktera colektomiutfall inom en månad.

Sammanfattningsvis har flera av markörerna i det här arbetet visat sig värdefulla i bedömningen av inflammatorisk aktivitet och skulle kunna utgöra ett viktigt verktyg i den kliniska vardagen.

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