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DIVISION OF GASTROENTEROLOGY, DEPARTMENT OF MEDICINE
HELSINKI UNIVERSITY CENTRAL HOSPITAL
HELSINKI, FINLAND

NONINVASIVE MONITORING OF ACTIVITY IN CROHN'S DISEASE

TAINA SIPPONEN

ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Medical Faculty of the University of Helsinki, in Auditorium 2 of the Haartman Institute, Haartmaninkatu 3, on the 15th of May 2009, at 12 noon

Helsinki 2009

Supervised by

Docent Martti Färkkilä, MD, PhD
Division of Gastroenterology, Department of Medicine
Helsinki University Central Hospital
Helsinki, Finland

Reviewed by

Docent Katri Kaukinen, MD, PhD
Medical School, University of Tampere and
Department of Gastroenterology and Alimentary Tract Surgery
Tampere University Hospital
Tampere, Finland

Docent Seppo Niemelä, MD, PhD
Division of Gastroenterology, Department of Medicine
Oulu University Hospital
Oulu, Finland

To be discussed with

Professor Walter Reinisch
Clinic of Internal Medicine III
Department of Gastroenterology and Hepatology
Medical University of Vienna
Vienna, Austria

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List of original publications

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I Sipponen T, Savilahti E, Kolho K-L, Nuutinen H, Turunen U, Färkkilä M. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: Correlation with Crohn's disease activity index and endoscopic findings. Inflammatory Bowel Diseases 2008;14(1):40-46.

II Sipponen T, Kärkkäinen P, Savilahti E, Kolho K-L, Nuutinen H, Turunen U, Färkkilä M. Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings. Alimentary Pharmacology & Therapeutics 2008;28(10):1221-1229.

III Sipponen T, Savilahti E, Kärkkäinen P, Kolho K-L, Nuutinen H, Turunen U, Färkkilä M. Fecal calprotectin, lactoferrin and endoscopic disease activity in monitoring anti-TNF-alpha therapy for Crohn's disease. Inflammatory Bowel Diseases 2008;14(9):1392-1398.

IV Hölttä T, Sipponen T, Westerholm-Ormio M, Salo H, Kolho K-L, Färkkilä M, Savilahti E, Vaarala O, Klemetti P. Anti-TNF-α treatment changes the balance between mucosal IL-17, Foxp3, and CD4 cells in patients with Crohn's disease. Submitted.

The publications are referred to in the text by their roman numerals, and reprinted by permission of the copyright holders.

Abbreviations

APC	antigen-presenting cell
ASCA	anti-*Saccharomyces cerevisiae* antibodies
CARD	caspase-activating recruitment domain
CBir1	anti-flagellin antibody
CD	Crohn's disease
CDAI	Crohn's disease activity index
CDEIS	Crohn's disease endoscopic index of severity
CI	confidence interval
CFAg	cystic fibrosis-associated antigen
CRP	C-reactive protein
CR-EDTA	Chromium-ethylene diaminetetraacetic acid
CT	computed tomography
DAMP	damage-associated molecular pattern protein
DBE	double-balloon enteroscopy
DPTA	diethylene triaminepentaasectic acid
EGD	esophagogastroduodenoscopy
ESR	erythrocyte sedimentation rate
ET	Edwards and Truelove score
F	fecal
Foxp3	forkhead transcription factor 3
g	gram
GI	gastrointestinal
HBI	Harvey Bradshaw index
HCT	hematocrit
kDa	kilo Dalton
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IEC	intestinal epithelial cell
IFN	interferon
IL	interleukin
MPO	myeloperoxidase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MRP	migration-inhibitory factor-related protein
NF-κB	nuclear factor kappa B
NOD	nucleotide oligomerisation domain
NPV	negative predictive value
NSAID	nonsteroidal anti-inflammatory drug
NK cell	natural killer cell
OMP-C	outer membrane porin C
pANCA	anti-neutrophil cytoplasmic antibody with perinuclear staining pattern
PCDAI	pediatric Crohn's disease activity index

PCR	polymerase chain reaction
PDAI	perianal Crohn's disease activity index
PEG	polyethylene glycol
PPV	positive predictive value
PRR	pattern-recognition receptor
R	receptor
RAGE	receptor for advanced glycation endproducts
S	serum
SES-CD	simple endoscopic score for Crohn's disease
SBE	small bowel enteroclysis
SBFT	small bowel follow-through
SD	standard deviation
TLR	toll-like receptor
TNF	tumor necrosis factor
UC	ulcerative colitis
WCE	wireless capsule endoscopy

Abstract

Background: Crohn's disease (CD), a transmural and segmental chronic bowel inflammation, can affect the entire gastrointestinal tract. In CD, symptoms sometimes fail to correlate with inflammation detected by endoscopy. Conventional laboratory markers such as serum C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) are insufficiently sensitive to reveal intestinal inflammation. The fecal neutrophil-derived proteins calprotectin and lactoferrin have proven useful surrogate markers of intestinal inflammation and can reliably distinguish between bowel inflammation and noninflammatory functional conditions. The correlation of these markers with CD endoscopic activity is, however, insufficiently examined. The aim of this study was to compare fecal calprotectin and lactoferrin concentrations to clinically, endoscopically, and histologically assessed CD activity. During tumor necrosis factor-alpha (TNFα) blocking therapy, we explored the suitability of these proteins as surrogate markers of mucosal healing. Furthermore, we studied changes in the number and expression of effector and regulatory T cells in bowel biopsy specimens during anti-TNFα therapy.

Patients and methods: Adult CD patients referred for ileocolonoscopy for various reasons were recruited. The number of endoscopies performed was 106 for 77 patients (Study I). Clinical disease activity was assessed with the Crohn's disease activity index (CDAI) and endoscopic activity with both the Crohn's disease index of severity (CDEIS) and the simple endoscopic score for Crohn's disease (SES-CD). Patients provided stool samples for measurements of calprotectin and lactoferrin, and blood samples for CRP. To explore correlations of fecal markers with histologic CD activity (Study II), we obtained biopsy specimens from the most severely affected lesions in the ileum and in four segments of the colon during 87 endoscopies on 61 patients. A histologic score was calculated based on the findings in the biopsy specimens.

Prospective Study III explored changes in endoscopic and clinical scores and fecal markers during anti-TNFα therapy for 15 adult CD patients. After baseline ileocolonoscopy, 14 patients received induction therapy with parenteral infliximab, and one patient received subcutaneous adalimumab. At the time of baseline endoscopy and 2, 8, and 12 weeks after the first treatment, each patient provided a diary for calculation of the CDAI, and samples for measurements of serum CRP, fecal calprotectin, and lactoferrin. Endoscopic and histologic responses to therapy were evaluated at 12 weeks after beginning of therapy. In Study IV, for further evaluation of alerations in mucosal inflammatory activity by detecting changes in the number and expression of effector and regulatory T cells, two biopsy specimens were taken from the most severely diseased lesions in the ileum and the colon during the baseline and post-treatment endoscopies. The control group comprised 14 patients without signs of endoscopic or histologic intestinal inflammation.

Results: CDEIS and SES-CD correlated significantly with fecal calprotectin (Spearman's rank order correlation coefficient $r=0.729$ for CDEIS and $r=0.699$ for SES-CD, both $p<0.001$) and lactoferrin ($r=0.773$ and $r=0.751$, both $p<0.001$). Correlations of CDEIS with CDAI ($r=0.381$) and CRP ($r=0.553$, both $p<0.001$) were weaker.

Both fecal markers were significantly lower in patients with endoscopically inactive (CDEIS <3) than with active disease (CDEIS ≥3) ($p<0.001$). In detecting endoscopically active disease, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for calprotectin ≥200 μg/g were 70%, 92%, 94%, and 61%; for lactoferrin ≥10 μg/g they were 66%, 92%, 94%, and 59%. Accordingly, the sensitivity, specificity, PPV, and NPV for CRP >5 mg/l were 48%, 91%, 91%, and 48%, and for CDAI with a cutoff 150 these were 27%, 94%, 91%, and 40%.

Fecal markers were significantly higher in active colonic (both $p<0.001$) or ileocolonic (calprotectin $p=0.028$, lactoferrin $p=0.004$) than in ileal disease. In ileocolonic or colonic disease, colon histology score correlated significantly with fecal calprotectin ($r=0.563$) and lactoferrin ($r=0.543$). In ileal disease, however, histology score failed to correlate with fecal markers ($r=0.311$ for calprotectin and $r=0.291$ for lactoferrin). Patients with normal fecal calprotectin or lactoferrin had significantly lower endoscopic ($p<0.001$) and histologic scores ($p<0.001$) than did those with elevated fecal markers.

In patients receiving anti-TNFα therapy, median CDEIS decreased from baseline level 13.0 (range 1.8-25.3) to 4.8 (0.0-11.2), $p=0.002$. Accordingly, fecal calprotectin decreased from 1173 μg/g (88-15326) to 130 μg/g (13-1419) and lactoferrin from 105.0 μg/g (4.2-1258.9) to 2.7 μg/g (0.0-228.5), both $p=0.001$. The CDAI fell from 158 (49-605) to 66 (24-202, $p=0.005$) and CRP from 10 mg/l (<5-54) to <5 mg/l (<5-6, $p=0.005$). Colon histology score declined from 23 (0-38) to 11 (0-25, $p=0.002$) and ileal histology score from 7 (0-11) to 4 (0-9, $p=0.022$).

Compared to controls, both before and after anti-TNFα therapy, the numbers of interleukin (IL) 17^+ cells and $Foxp3^+$ cells in ileal specimens were significantly increased (all $p<0.05$). Accordingly, in the colon, the number of IL-17^+ cells was increased before and after the therapy ($p=0.015$ and $p=0.042$), and $Foxp3^+$ cells were increased compared to control numbers only after the treatment ($p=0.032$). Changes in the number of IL-17^+ cells and $Foxp3^+$ cells during therapy were nonsignificant, but the relation of ileal IL-17^+ cells to $CD4^+$ cells decreased significantly during anti-TNFα treatment ($p=0.047$). The relation of IL-17^+ cells to $Foxp3^+$ cells was higher in the patients' baseline specimens than in their post-treatment specimens ($p=0.038$).

Conclusions: For evaluation of CD activity, based on endoscopic findings, more sensitive surrogate markers than CDAI and CRP were fecal calprotectin and lactoferrin. Fecal calprotectin and lactoferrin were significantly higher in endoscopically active disease than in endoscopic remission. In both ileocolonic and colonic disease, fecal markers correlated closely with histologic disease activity. In CD, these neutrophil-derived proteins thus seem to be useful surrogate markers of endoscopic activity.

During anti-TNFα therapy, fecal calprotectin and lactoferrin decreased significantly. Furthermore, endoscopically confirmed mucosal healing was associated with reduced mucosal IL-17/CD4 cell ratio. The anti-TNFα treatment was also reflected in a decreased IL-17/Foxp3 cell ratio, which may indicate improved balance between effector and regulatory T cells with treatment.

Introduction

Crohn's disease (CD), ulcerative colitis (UC), and colitis unclassified, all being the consequence of chronic inflammatory reactions in the gastrointestinal tissue, are collectively defined as inflammatory bowel disease (IBD). These are chronic diseases arising from interactions between immunoregulatory, genetic, and environmental factors. While UC is limited to the colon, CD can affect the whole gastrointestinal tract.

In CD, symptoms do not always correlate with inflammatory activity in the bowel: Patients with highly active inflammation may be nonsymptomatic or may have adapted to their chronic symptoms, and some patients may show symptoms regardless of mucosal healing. In these situations, endoscopy may help in treatment decisions. Endoscopy with biopsies is the mainstay in the diagnosis of both CD and UC and important in the assessment of disease activity and monitoring of treatment. New endoscopy techniques of the intestine such as wireless capsule endoscopy now allow direct and objective assessment of mucosal healing. Mucosal healing is becoming a target for new CD therapies that associate with better outcome of the disease with fewer hospitalizations or need for surgery. However, due to the costs and to limited endoscopic capacity, the need is strong for reliable surrogate markers to monitor disease activity. Several clinical activity scores are widely used, but they are subjective and often underestimate the inflammation. Conventional laboratory tests such as hemoglobin, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) are insufficiently sensitive to detect intestinal inflammation.

Bowel contents are in close contact with intestinal mucosa and can take up molecules that are measurable from stool samples and thus serve as markers of inflammation. The fecal neutrophil-derived proteins calprotectin and lactoferrin have several features of an ideal test for detecting inflammation: They are nonivasive, simple, and low in cost. They allow serial monitoring of disease activity and of treatment success, and can even serve in predicting clinical relapse in nonsymptomatic patients. The utility of these markers in distinguishing IBD from non-inflammatory conditions such as irritabile bowel syndrome is well documented. Correlation of these markers with validated endoscopic scores in CD is, however, insufficiently studied.

This thesis aims to determine the correlation of fecal calprotectin and lactoferrin with endoscopic, histologic, and clinical disease activity in CD and the suitability of these fecal proteins as surrogate markers of disease activity. Another aim was to study how these fecal markers serve as surrogate markers of treatment monitoring in CD compared to endoscopy and histology. Finally, during anti-tumor necrosis factor alpha (TNFα) therapy, focusing in interleukin-17 (IL-17)-secreting cells, we studied mucosal effector and regulatory T-cell balance, which may reflect intestinal inflammatory activity.

Review of the literature

Crohn's disease— also called terminal ileitis, regional enteritis, granulomatous ileitis, hyperplastic ileitis, chronic ulcerative ileitis, and intestinal phlegmon—is named after the American Dr. Burrill Crohn, who with his colleagues Ginzburg and Oppenheimer described regional enteritis over 75 years ago (Crohn *et al* 1932). CD is a transmural inflammatory disease of the gastrointestinal mucosa that can affect any part of the entire gastrointestinal (GI) tract (Baumgart and Sandborn 2007). It is characterized by a chronic course in which phases of remission are interrupted by unpredictable acute episodes (relapses). Some patients may have continously active inflammation (chronically active disease).

A slight female preponderance in CD occurs, depending however, on age and geographic region (Brant and Nguen 2008). CD typically manifests in young adolescents, and approximately 20% of all IBD patients develop symptoms in childhood (Heyman *et al* 2005, Nikolaus and Schreiber 2007). The highest incidence rates and prevalences occur in developed countries with a yearly incidence of up to 16.3/100 000 and a prevalence of 213/100 000 (Loftus 2004, Lapidus 2006, Baumgart and Carding 2007, Bernstein and Shanahan 2008). One estimates that annually Europe has 23,000 to 41,000 new CD cases (Loftus 2004). A distinct north-south gradient occurs within Europe, but the incidence in southern Europe has increased in recent years (Shivananda *et al* 1996). At diagnosis, age below 40 years, presence of perianal disease, and initial need for corticosteroid therapy are factors predictive of a more disabling disease course (Beaugerie *et al* 2006). The life-expectancy of patients with CD seems to be slightly below average (Jess *et al* 2002).

1 Etiology and pathogenesis of Crohn's disease

Although the understanding of immunological mechanisms has developed markedly during recent years, CD etiology and pathogenesis is not fully known. The most widely accepted hypothesis is that a disturbed interaction of the host immune system with its commensal microflora and other luminal antigens leads to damage of the bowel mucosa (Baumgart and Carding 2007, Brown and Mayer 2007).

1.1 Genetics

An inherited predisposition is important in the pathogenesis of CD, with CD more prevalent in Jewish people than in any other ethnic group. According to three large twin studies, the pooled concordance is 36% in monozygotic twins (Tysk *et al* 1988, Orholm *et al* 2000, Thompson *et al* 1996, Russell and Satsangi 2004). Patients have a first-degree relative with IBD in 2 to 22% of cases (Gaya *et al* 2006). The discovery of the gene encoding caspase-activating recruitment domain 15 (CARD15)—also known as the nucleotide oligomerisation domain 2 (NOD2)—has been important in the understading of

interactions between genetic factors and bacterial flora (Hugot *et al* 2001, Gaya *et al* 2006). It plays a key role in innate host defense, and mutations of this gene are strongly associated with ileal CD and especially with stricturing disease. However, ethnic variation in the contribution of this gene occurs; in Europe, allelic frequency is lower in northern countries (about 5% in Finland) than in central or southern Europe (9-14%) (Heliö *et al* 2003, Gaya *et al* 2006). Furthermore, recent studies have revealed that several polymorphisms of the IL-23 receptor (IL-23R) gene locus are associated with CD susceptibility (Duerr *et al* 2006). Recently, a genome-wide association study combining data of three studies defined more than 30 distinct CD susceptibility loci (Barrett *et al* 2008).

1.2 Environmental factors

Several environmental factors such as diet, bacteria, viruses, use of nonsteroidal antiinflammatory drugs (NSAIDs), and high hygiene level have been studied as CD triggers. Of life style factors, the effect of smoking in CD is best documented: it adversely affects the course of the disease, raises exacerbation rates, promotes complications and risk for surgery (Johnson *et al* 2005).

1.3 Role of immune response

Mucosal host defense can be divided into innate immune response and adaptive immune response. The innate immune system is a nonspecific defense against pathogens by the macrophages, dendritic cells, natural killer (NK) cells, neutrophils, and the complement system. Responses of these components are inborn and not tailored to a particular immunological challenge. In the adaptive immune system, the main cells are T and B lymphocytes. Adaptive immune response is a slower and secondary response and is more specifically tailored through function of these cells. In CD, abnormalities occur in the mucosal barrier, innate immune response, and adaptive immune system.

A defect in the intestinal mucosal barrier can lead to loss of tolerance to commensal microbial flora. In the healthy gut, the mucosal barrier—consisting of secreted and cell-surface mucins and a single layer of intestinal epithelial cells (IECs)— protects the mucosal immune system (Brown and Mayer 2007). Secreted IgA from mucosal plasma cells coats and neutralizes pathogens, and IECs can secrete a number of cytokines and chemokines. Defensins and cryptidins secreted from Paneth cells in the small intestine act as antimicrobial agents preventing microbial invasion into the crypt microenvironment. In ileal CD, expression of α-defensins is reportedly reduced compared to that of unaffected ileum and to the ileum from healthy controls (Wehkamp *et al* 2007).

Antigen-presenting cells (APCs) such as dendritic cells express a wide spectrum of pattern-recognition receptors (PRRs). Two types of PRRs are involved in this host response to microbes in the intestinal tract: membrane-associated toll-like receptors (TLRs) and cytosolic NOD proteins. In healthy IECs, TLR3 and TLR5 are constitutively expressed

and TLR2 and TLR4 barely detectable, whereas in active CD, TLR3 is downregulated and TLR4 is overexpressed (Baumgart and Carding 2007). NOD2, which is preferentially expressed in macrophages, dendritic cells, and Paneth cells, recognizes muramyl dipeptide, the smallest structure of bacterial wall peptidoglycan, and mediates the interaction between the innate immune response and the host. Mutations in the NOD2 gene are associated with a decrease in nuclear factor-κB activation (NF-κB), leading to an inappropriate response to muramyl dipeptide (Inohara *et al* 2003).

In IBD, dendritic cells may falsely recognize commensal bacteria as pathogens and are activated, promoting differentiation of naïve T cells (Th0) into effector T cells (Th1, Th17, and Th2) and natural killer T cells. In CD, naïve T cells preferably differentiate into Th1, producing interferon-γ (IFN-γ) and IL-12, whereas in UC, differentiation of Th0 cells into Th2 cells predominates. IL-23, produced by innate immune cells, activates Th17-type T cells and seems to be imporant in maintaining Th17 cell responses (Caprioli *et al* 2008). In active inflammation, effector T cells predominate over regulatory T cells. These activated effector cells secrete proinflammatory cytokines and stimulate macrophages to secrete large amounts of cytokines such as TNFα, IL-1, and IL-6. Furthermore, defects in regulatory T-cell function and in T-cell apoptosis can occur (Brown and Mayer 2007). A marker for regulatory T cells is the forkhead transcription factor Foxp3 expressed mainly in regulatory T cells. The Foxp3 transcription seems to be upregulated both in active disease and in CD remission (Hölttä *et al* 2008).

Neutrophils are among the body's main cellular components for the destruction of microorganisms. The majority of neutrophils is storaged in the bone marrow. In response to inflammatory messengers such as cytokines, neutrophils are released from the bone marrow into the blood stream and access inflamed tissues. Neutrophils, important contributors to recruitment and activation of APCs, cause tissue damage through the release of nonspecific inflammatory mediators and secrete cytokines and multiple anti-microbial agents such as calprotectin, lactoferrin, neutrophil elastase, myeloperoxidase, and lysozyme (Nathan 2006). In CD, changes in neutrophil function include defective neutrophil migration due to impaired chemotaxis (Harbord *et al* 2006). Furthermore, impaired apoptosis of neutrophils can result in an extended lifespan for these cells and may promote tissue damage (Brown and Mayer 2007).

2 Disease location and classification by phenotype

At diagnosis, in approximately 25% of patients both the terminal ileum and colon are affected, about 45% show exclusively terminal ileitis, and in about 25% only the colon is involved (Louis *et al* 2001, Baumgart and Sandborn 2007). In up to 10% of CD patients the disease may affect the ileum out of reach of ileocolonoscopy or involve the more proximal small bowel (Stange *et al* 2006). Small intestinal and upper GI disease is more common in patients diagnosed before age 20, whereas colonic disease is diagnosed more frequently in late-onset (>40 years) disease (Polito *et al* 1996). Interestingly, in contrast to older children or adults, almost all children diagnosed before age eight have colonic

disease (isolated or in combination to small bowel disease) (Heyman *et al* 2005, Silverberg *et al* 2005).

Typical presentations include discontinous involvement of various portions of the GI tract and development of disease complications such as strictures, fistulas, or abscesses. The Vienna classification has been developed to describe the distinct clinical CD phenotypes with respect to disease location and occurrence of complications (Gasche *et al* 2000). Age at diagnosis (A: A1 <40 years, A2 ≥40 years), disease location (L: L1 ileum, L2 colon, L3 ileocolon, L4 upper GI), and disease behavior (B) are three categories for allocating patients. Disease behavior is classified as non-stricturing and non-penetrating (inflammatory, B1), stricturing (B2), or penetrating (B3) (Gasche *et al* 2000). Anatomical location remains relatively stable over the course of the disease, but disease behavior may change over time, with the most prominent change being from inflammatory to either stricturing or penetrating (Louis *et al* 2001, Cosnes *et al* 2002). According to a Norwegian 5-year follow-up study of new CD cases, however, changes in disease location were apparent at 5 years in 13.5% and in disease behavior in 17.5% of patients (Henriksen *et al* 2007). Stricturing complications were evident in 64% of ileal CD patients but in only 6% of patients with colonic CD.

An update of the Vienna classification was proposed in Montreal, including a revised age category and upper GI tract and perianal disease modifiers for disease location and behavior (Silverberg *et al* 2005, Satsangi *et al* 2006). "Upper GI tract" in the classification means any disease proximal to the terminal ileum.

3 Diagnosis

No single specific test exists for Crohn's disease. Lennard-Jones and Shivananda with the European IBD Study Group (1997) have defined widely accepted criteria for diagnosis of CD, including macroscopic discontinuity of disease, transmural inflammation (shown by fistula or abscess formation outside the gut or by examination of a surgical specimen), fibrosis (evidenced by stricture formation), and histologic criteria (lymphoid aggregates, discontinuous inflammation, or granulomas). According to these criteria, CD is established if granuloma with at least one criterion is present, or, in the absence of granuloma, if three criteria are present. CD is probable with two criteria without granulomas (Lennard-Jones and Shivananda 1997). These criteria seem, however, too strict in early CD and may be biased towards complicated CD (Reinisch 2007). The current view is that diagnosis is established by a broadly defined combination of clinical presentation, endoscopic appearance, histology, radiology, and surgical findings (Stange *et al* 2006, Baumgart and Carding 2007).

3.1 Clinical presentation

Clinical presentation depends on disease location. Chronic diarrhea is the most common symptom, affecting up to 85% of patients (Nikolaus and Schreiber 2007), with abdominal

pain occurring in about 70% and weight loss in about 60% (Lennard-Joes and Shivananda 1997). Fever, rectal pain, and fatigue can also be present. Blood or mucus or both can be present in stools in approximately 50% of patients and perianal fistulas in 10% at diagnosis (Stange *et al* 2006). Children can present with growth retardation. A considerable number (up to 30%) of IBD patients may suffer from extraintestinal manifestations such as peripheral arthropathy, axial arthritis, ocular (uveitis, episcleritis), cutaneous (erythema nodosum, pyoderma gangrenosum), or hepatobiliary disease (primary sclerosing cholangitis) (Stange *et al* 2006).

In physical examination, a CD patient may appear underweight. Inspection of the oral mucosa may reveal small aphthous ulcers. Abdominal bowel sounds may be abnormal, and on palpation, pain or abnormal mass detectable. Patients with ileal involvement can present with pain in the right lower abdomen. Evidence of perianal fistulae or fissures can be detectable upon inspection of the anal region, and gross abnormalities of the rectal mucosa and hematochezia detectable in rectal examination.

In diagnostic work-up, stool tests for investigation of pathogenic bacteria and parasites are necessary to differentiate IBD from infectious colitis. Blood tests may reveal anemia or elevated leukocyte and platelet counts. Serum CRP can be elevated and hypoalbuminemia can be present.

3.2 Endoscopy

The mainstay for IBD diagnosis is ileocolonoscopy. Initial endoscopy should intubate the terminal ileum, and in practice ileocolonoscopy with biopsies has been achieved in at least 85% of patients. Inflamed mucosal areas alternating with noninflamed mucosal surfaces ("skip lesions") is a typical finding. The rectum can be spared. Small, deep aphthous ulcers or longitudinal, polygonal ulcers are characteristic configurations. In chronic disease, a patchy, cobblestone pattern is often visible in the terminal ileum (Nikolaus and Schreiber 2007), which is a typical location for strictures. Fistulas, fissures, or anal skin tags can be evident. Ileocolonoscopy is time-consuming, expensive, requires bowel preparation, and is unpleasant for patients. When severe active inflammation is present, initial flexible sigmoideoscopy may be safer (due to risk for bowel perforation), with ileocolonoscopy postponed until clinical condition is improved.

Esophagogastroduodenoscopy (EGD) is often in clinical practice reserved for CD patients with upper GI symptoms, but some experts suggest that it should be performed at least once on all newly diagnosed CD patients (Hommes and van Deventer 2004). EGD can be useful in patients with their colitis unclassified: Focal active gastritis in the absence of ulceration may be a CD feature (Stange *et al* 2006).

Radiographic techniques were formerly the only available techniques for examination of the entire small bowel, but during recent years, small bowel wireless capsule endoscopy (WCE), double-balloon enteroscopy (DBE), and magnetic resonance imaging (MRI) enterography have made radiation-free examination of the whole small bowel possible. WCE, a novel technique for examining the entire small bowel and directly visualizing small bowel lesions, is useful in suspicion of small bowel CD and in assessment of small

bowel CD extension and severity. In patients with unclassified colitis, WCE may help in distinguishing between UC and CD. WCE is superior to small bowel follow-through (SBFT), barium enteroclysis (SBE), and conventional computed tomography (CT) in establishing the diagnosis and estimating disease extent and is widely considered a first-line examination after negative colonoscopy and EGD (Sandrasegaran *et al* 2008). In many centers, WCE has replaced more invasive push enteroscopy, but WCE is limited by its cost and its inability to provide tissue samples or therapy. Furthermore, with WCE, localization of lesions is troublesome. Contraindications for WCE are suspected or documented intestinal obstruction or strictures.

Double–balloon enteroscopy is a new technique that makes it possible to reach lesions throughout the entire small bowel (Yamamoto *et al* 2001). The scope may be inserted by either an oral or anal route. DBE is not widely available, and its use in CD is at present mainly therapeutic, i.e., dilation of strictures.

3.3 Imaging techniques

Due to its ubiquitous availability, barium studies SBFT and intubation technique-requiring SBE are still considered the current standards for assessment of small bowel CD (Stange *et al* 2006), although they cause a radiation burden and are therefore not suitable for follow-up. Compared to SBFT and SBE, cross-sectional CT gives additional information on the bowel wall and adjacent structures. CT-enteroclysis is superior to conventional enteroclysis in depicting CD-associated intra- and extramural abnormalities (Engin 2008), and quantitative measures of bowel enhancement at CT-enterography correlate with endoscopy and histology in ileal CD (Colombel *et al* 2006). Because of its radiation burden, CT is unsuitable for repeated use. In some centers either MRI-enterography or MRI-enteroclysis has already replaced radiation techniques in assessment of small bowel CD. These procedures can be performed repeatedly and are thus suitable for follow-up of CD patients. Small bowel MRI provides information on disease activity and detects extramural complications (abscesses, fistulas, sacroilitis). In detecting inflammatory small bowel changes, the sensitivity (95%) and the specificity (93%) of MRI-enteroclysis are superior to those of SBE (85% and 77%) (Rieber *et al* 2000); also in assessment of perianal fistulas MRI is a sensitive technique and in the setting of clinical trials is considered mandatory (Caprilli *et al* 2006). Ultrasound may be of benefit in detecting ileal disease and extramural complications of CD, but does not provide information on the extent of the disease and is operator-dependent (Stange et al 2006).

3.4 Histology

Histologic examination is routinely used for the diagnosis of IBD and is helpful in the histologic distinction between UC and CD. In UC, inflammation is limited to the colon and is superficial, whereas in CD it is generally transmural, multifocal, and may contain granulomas.

For the initial diagnosis of CD, during ileocolonoscopy multiple mucosal biopsies should be obtained from all segments of the colon (right, transverse, the left colon and sigmoid, and rectum) and the ileum. Biopsy samples preferably come both from areas affected by the disease and from areas unaffected (Stange *et al* 2006). Typical histologic changes in CD in mucosal biopsy specimens are focal (segmental or discontinuous) crypt architectural abnormalities in conjunction with focal or patchy chronic inflammation (presence of lymphocytes or plasma cells), granulomas, and mucin preservation at active sites (Jenkins *et al* 1997, Stange *et al* 2006). The same features and additionally an irregular villous architecture can serve in analysis of ileal biopsy samples. Granulomas—defined as a collection of epitheloid histiocytes—are regarded as a corroborating feature of CD when present in the lamina propria without association with active crypt injury (Mahadeva *et al* 2002). Crypt-associated granulomas can occur also in UC and are a less reliable feature in discrimination between UC and CD (Mahadeva *et al* 2002). The presence of granuloma is also the central histologic criterion among Lennard-Jones criteria (Lennard-Jones and Shivananda 1997). The transmural character of CD inflammation can be identified only when surgical samples are available. Other microscopic features detectable in surgical specimens of CD patients are aggregated inflammatory pattern, transmural lymphoid hyperplasia, submucosal thickening, fissures, sarcoid granulomas (including in lymph nodes), abnormalities of the enteric nervous system (submucosal nerve fiber hyperplasia and ganglionitis), and relatively unchanged epithelial mucin preservation (Stange *et al* 2006).

4 Treatment

When treating active CD, a clinician has to consider the disease activity, site (ileal, ileocolonic, colonic, or other), and disease behavior (inflammatory, stricturing, or fistulating). Seldom, in mild disease, is no treatment an option. Smoking cessation is associated with a 65% reduction in risk for relapse and should be encouraged in all smokers with CD (Cosnes *et al* 2001, Johnson *et al* 2005).

4.1 Medical therapy

Medical therapy for CD can be divided into induction and maintenance of remission.

4.1.1 Corticosteroids

Use of corticosteroids in CD is based on two placebo-controlled trials showing the effectiveness of oral corticosteroids in induction of clinical remission (Summers *et al* 1979, Malchow *et al* 1984, Irving *et al* 2007). Most IBD patients initially respond to the first

course of corticosteroid therapy, but about half will be either steroid-resistant or steroid-dependent at one year (Munkholm *et al* 1994, Faubion *et al* 2001). Importantly, mucosal healing is achieved in only about a third of CD patients treated with corticosteroids (Modigliani *et al* 1990, Landi *et al* 1992). Corticosteroids play no role in maintaining remission.

In mildly active ileocecal CD, budesonide is the preferred treatment (Greenberg *et al* 1994, Travis *et al* 2006). This corticosteroid has an effective first-pass hepatic metabolism, and its pH- and time-dependent formulation enables a targeted release to the terminal ileum and right colon. Budesonide is associated with fewer systemic side-effects than are systemic corticosteroids. In moderately active disease, induction of remission can be achieved with either budesonide (ileal and ileocecal CD or disease limited to the proximal colon) or systemic corticosteroids. Severely active ileocecal disease or moderate to severe extensive small bowel CD should be treated initially with systemic corticosteroids (Travis *et al* 2006).

4.1.2 Mesalamine, sulfasalazine, and antibiotics

According to one meta-analysis, the benefit of 5-aminosalicylic acid (5-ASA) mesalamine in induction and maintenance of CD remission is limited (Hanauer and Stromberg 2004). However, it may prove of some benefit in the postoperative treatment of small intestinal resection (Caprilli *et al* 2006). In mildly active colonic disease, especially when associated with arthropathy, sulfasalazine can be effective for induction of clinical remission, but its use is limited due to sulfa-related intolerance (Summers *et al* 1979). Some selected patients with colonic disease can response to metronidazole, but it is not considered first-line therapy (Sutherland *et al* 1991). When combined with azathioprine, metronidazole may be useful in selected patients in prevention of postoperative recurrence (D´Haens *et al* 2008a). Antibiotics are in general considered appropriate in septic complications, in perineal disease, or in symptoms due to bacterial overgrowth (Travis *et al* 2006).

4.1.3 Immunomodulators

Immunomodulators should be started in corticosteroid-dependent or corticosteroid-refractory disease or in extensive small bowel CD (Travis *et al* 2006). Thiopurines (azathioprine and 6-mercaptopurine) and methotrexate are immunomodulatory medications. A Cochrane analysis has shown the benefit of thiopurines compared with placebo in inducing remission in active CD (Sandborn *et al* 1998). Azathioprine has proven efficacious in the maintenance of remission in an analysis reviewing five clinical trials (Pearson *et al* 1998). As the onset of action is delayed, thiopurines are suitable for CD maintenance treatment, but not for a rapid induction of remission. In both ileal and colonic CD, azathioprine therapy can lead to mucosal healing (D'Haens *et al* 1997, 1999a). Methotrexate has been less studied than azathioprine, but mucosal healing seems to occur during intramuscular methotrexate therapy (Kozarek *et al* 1989). In placebo-controlled

trials, methotrexate showed effectiveness in both inducing and in maintaining CD clinical remission (Feagan *et al* 1995, 2000).

4.1.4 TNFα-blocking agents

Infliximab is an intravenously adminstered chimeric immunoglobulin G1 monoclonal antibody against TNFα that has been shown to induce both rapid clinical remission and endoscopic healing in CD (D'Haens *et al* 1999b, Travis *et al* 2006). By 10 weeks, repeated infliximab infusions (0, 2, and 6 weeks) resulted in complete mucosal healing more often (29% versus 3%) than did only one baseline infusion (Rutgeerts *et al* 2004). At one year, systematic treatment (scheduled infusions every 8 weeks) has been superior to episodic treatment in inducing clinical remission (Hanauer *et al* 2002) and complete mucosal healing (Rutgeerts *et al* 2004). It seems that mucosal healing is associated with fewer hospitalizations and surgeries, but the data are as yet limited (Rutgeerts *et al* 2004). In recently diagnosed CD patients, early treatment with combined immunosupression (infliximab and azathioprine or methotrexate) seems to be more effective than conventional management for induction of remission or reduction of corticosteroids through 52 weeks (D'Haens *et al* 2008b). Infliximab is indicated in moderate to severe corticosteroid-refractory or -dependent, or in immunomodulator-refractory disease or intolerance (Travis *et al* 2006). It is also effective in the induction and maintenance of perianal or abdominal fistulating CD (Sands *et al* 2004).

Adalimumab is a fully human anti-TNFα monoclonal antibody administered by subcutaneous injections usually every other week. Adalimumab has been effective in inducing clinical remission in both TNF-naïve patients and in those patients who have lost response or are intolerant to infliximab (Hanauer *et al* 2006, Sandborn *et al* 2007a). Through week 56, adalimumab has been superior to placebo in maintaining remission in initial responders (Colombel *et al* 2007). Data on mucosal healing during adalimumab treatment are, however, as yet limited.

Certolizumab, a pegylated humanized TNFα-binding Fab´fragment, is administered subcutaneously. In a placebo-controlled trial, it showed only a modest response in moderate to severe CD and led to non-significant improvement in remission rates (Sandborn *et al* 2007b). Patients who responded to induction therapy with certolizumab were more likely to maintain the response and sustain remission at 26 weeks with continous certolizumab than did those switched to placebo (Schreiber *et al* 2007).

Under research are several biological therapies targeted to mechanisms other than blockade of TNF, including modulation of other cytokines, blockade of T cells, and blockade of inflammatory cell migration and adhesion. The blockade of α4-integrin with natalizumab showed promising results in treatment of CD, but it is not available in Europe because of reported severe adverse effects (Baumgart and Sandborn 2007).

4.2 Operative treatment

Although surgery in CD is limited to complications of CD such as strictures and fistulas, it is often needed as part of the treatment. In a cohort of 592 CD patients diagnosed from 1966 to 1969 and observed for at least 7 years, 225 (91%) ileocolonic CD patients, 108 (65%) with small bowel disease, and 105 (58%) with colonic or anoperineal disease underwent a surgical procedure (Farmer *et al* 1985). According to Loftus *et al* (2002), 40 to 60% of CD patients need at least one operative procedure. During a 5-year follow-up in Norway, 28% of CD patients underwent surgery with intestinal resection, and half of these had disease limited to the terminal ileum (Henriksen *et al* 2007). According to a retrospective trial, need for surgery has not decreased significantly over the last 25 years, although immunosuppressant use has been more frequent (Cosnes *et al* 2005). In northern Europe, CD patients seem more likely to be treated with surgery than in southern Europe suggesting a disease-severity gradient across Europe (Wolters *et al* 2007).

Specific indications for surgery include symptomatic fibrostenotic strictures, enterovesical or enterocutaneous fistulas, and enteral fistulas leading to abscesses (Larson *et al* 2004). Perforation of the bowel or severe bleeding may necessitate surgical treatment. Perianal or rectovaginal fistulas often need a combination of surgical and medical treatment. Small bowel resection should be as bowel-sparing as possible. Strictureplasty can be performed on isolated strictures under 10 cm in length. Symptomatic relief is achieved in the majority of patients, with second surgery rates of between 34 and 44% during a 7-year follow-up (Larson *et al* 2004).

4.3 Nutritional therapy

Nutritional support is often required in severe CD for treatment of malnutrition. Enteral feeding has been effective in CD, but it requires high patient motivation, can be unpalatable, and is costly. In adolescents refractory or intolerant to corticosteroid therapy and with growth failure it may become the treatment of choice (King *et al* 1997). In one recent study, a long-term enteral nutrition in quiescent CD seemed to result in a suppressive effect on clinical and endoscopic disease activity and on mucosal cytokine levels (Yamamoto *et al* 2007).

5 Assessment of disease activity

5.1 Clinical activity

Various activity indices have been developed to standardize and quantify disease severity. In clinical trials, the score most commonly used is the Crohn's disease activity index (CDAI) which comprises eight clinical variables (Table 1) (Best *et al* 1976). CDAI scores range from 0 to approximately 650. A CDAI <150 has been the limit for clinically inactive

disease, and for severe disease the cutoff value has been 450. Furthermore, some investigators have arbitrarily labeled CDAI scores of 150 to 219 as mildly, and 220 to 450 as moderately active disease (Sostegni *et al* 2003). Clincal response is suggested to be defined as ΔCDAI ≥-100 points, although some clinical trials have used ΔCDAI ≥-70 for response (Stange *et al* 2006).

The CDAI score is not actively used in everyday clinical work because of its rather complex calculation and the need for a 7-day diary of symptoms. Further, it is not useful in patients with extensive previous ileocolonic resection or stoma and it is not accurate in patients having symptoms mainly due to fistulating and stenosing behavior. A limitation of the CDAI score is also a relevant weight for scores of "general well-being" and "intensity of abdominal pain" items, which are subjective and reflect patients' perceptions of their disease (Sostegni *et al* 2003). For scoring of clinical disease activity of children, a pediatric Crohn's disease activity index (PCDAI) has been developed (Hyams et al 1991).

Table 1. Crohn´s Disease Activity Index (CDAI) (Best *et al* 1976).

Variable	*Description*	*Multiplier*
Number of liquid stools	sum of 7-day numbers	x2
Abdominal pain	sum of 7-day scores, ratings from 0 to 3 (none =0, mild =1, moderate =2, severe =3)	x5
General well-being	sum of 7-day scores, ratings from 0 to 4 (generally well =0, slightly poor =1, poor =2, very poor =3, terrible =4)	x7
Extraintestinal complications	number of listed complications (arthritis/arthralgia, iritis/uveitis, erythema nodosum, pyoderma gangrenosum, aphtous stomatitis, anal fissure/fistula/abscess, fever >37.8°C)	x20
Antidiarrheal drugs	use in the previous 7 days (no =0, yes =1)	x30
Abdominal mass	no =0, questionable =2, definite =5	x10
Hematocrit (Hct)	females 42-observed Hct; males 47-observed Hct	x6
Body weight	ideal/observed ratio [1-(ideal/observed)] x 100	x1 (not < -10)

A summary of different clinical activity indices is presented in Table 2. A simple index or Harvey Bradshaw index (HBI) is often used in clinical trials; it is based on five variables (Harvey and Bradshaw 1980) and correlates closely with the CDAI. Clinical remission is usually assessed as a HBI less than four or five (Tibble et al 2000a, Kane *et al* 2003). A modification of HBI is suggested to limit the effect of bowel movement number on total score (by scoring this variable on a scale from 0 to 5) (Myren *et al* 1984). A meeting of the International Organisation for the Study of Inflammatory Bowel Disease in Oxford proposed an index based on nine clinical and one hematological parameter (Myren *et al*

1984). The Cape Town index, also proposed for assessment of clinical activity, and the Oxford index correlate with the CDAI (Myren *et al* 1984, Wright *et al* 1985, Sostegni *et al* 2003), whereas the prospectively validated van Hees or Dutch index—a combined clinical and laboratory index—correlates poorly with the CDAI (van Hees *et al* 1980, Sostegni *et al* 2003). Compared to the HBI and van Hees indices, the CDAI has the highest variation, and self-reported wellness contributed 40% to total variance in the CDAI (Jorgensen *et al* 2005).

The CDAI in fistulating CD represents poorly the activity of perianal disease or CD with other fistulas. The perianal disease activity index (PDAI) represents at present the gold standard for evaluating perianal disease severity (Sostegni *et al* 2003).

Table 2. Various clinical activity indices for Crohn's disease.

Activity index	*reference*	*variables*
CDAI	Best *et al* 1976	diarrhea, abdominal pain, general well-being, extraintestinal manifestations, use of antidiarrheal medications, presence of abdominal mass, hematocrit, weight
HBI	Harvey and Bradshaw 1980	number of liquid stools, abdominal pain, general well-being, extraintestinal manifestations, abdominal mass
van Hees index (Dutch index)	van Hees *et al* 1980	serum albumin, ESR, Quetelet index (weight x 10/height x height), abdominal mass, gender, temperature, stool consistency, previous resection, extraintestinal lesions
Oxford index	Myren *et al* 1984	pain, bowel action or blood and mucus, perianal complications, fistula, other complications, abdominal mass or tenderness, wasting, temperature, hemoglobin
Cape Town index	Wright *et al* 1985	abdominal pain, diarrhea or blood and mucus, well-being, complications (perianal or systemic), abdominal mass and tenderness, weight, temperature, hemoglobin
PCDAI	Hyams *et al* 1991	abdominal pain, diarrhea, general well-being, weight and height, abdominal findings (tenderness and mass), perirectal disease, extraintestinal manifestations, hematocrit, ESR, s-albumin
PDAI	Irvine 1995	discharge of fistulas, pain/restriction of activities, restriction of sexual activity, type of perianal disease, degree of induration

CDAI, Crohn's disease activity index, HBI, Harvey-Bradshaw index; ESR, erythrocyte sedimentation rate; PCDAI, pediatric Crohn's disease activity index; PDAI, perianal disease activity index.

5.2 Endoscopic activity

Endoscopy with biopsies is the current gold standard for assessing intestinal inflammation. In addition to diagnostics, clear-cut indications for endoscopy are assessment of the disease activity and extension, dilation of strictures, and surveillance of long-standing chronic colonic disease (Hommes and van Deventer 2004).

Follow-up endoscopies are needed when uncertainty exists about disease activity or disease location. The location of inflammation is relevant for the choice of treatment (systemic or topically active). In CD, blood tests and symptoms do not necessary correlate accurately with endoscopic disease activity (Cellier *et al* 1994). When activity is measured by fecal excretion of ^{111}In-labeled granulocytes, intestinal inflammation is often present in symptomless patients (Saverymutty 1986). On the other hand, symptoms compatible with irritable bowel syndrome (IBS) can sometimes dominate the clinical picture despite IBD remission and can be two to three times as prevalent in IBD as in the non-IBD population (Simren *et al* 2002).

Assessment of mucosal healing during therapy is relevant for clinical practice and also for response evaluation during clinical trials (Hommes and van Deventer 2004, Rutgeerts *et al* 2007). For treatment of IBD, recent studies and reviews suggest mucosal healing as a therapeutic target (Arnott *et al* 2002, Froslie *et al* 2007). Total disappearance of mucosal ulcerations has served in infliximab trials as the definition of complete mucosal healing (Rutgeerts *et al* 2004, 2006), but the significance of subtle changes (for example an aphthous ulcer) in otherwise healed mucosa remains unknown. The optimal timing for detection of mucosal healing remains undetermined (Rutgeerts *et al* 2007). Furthermore, endoscopy fails to detect disease activity beyond the mucosa. For detection of transmural disease activity, the imaging techniques, decribed in section 3.3 may be useful.

5.2.1 Crohn's disease index of severity (CDEIS)

To measure endoscopic disease activity of CD, the French group GETAID (Groupe d´Etude Therapeutique des Afflections Inflammatoires Digestive) developed the Crohn's disease index of severity (CDEIS) (Mary *et al* 1989). At present it represents the gold standard for evaluation of endoscopic CD activity (Table 3) (Sostegni *et al* 2003). For calculation of the CDEIS, the intestine is divided into five segments: rectum, left colon and the sigmoid, transverse, and right colon, and the ileum; nine mucosal lesions are recorded from each studied segment: pseudopolyp, healed ulceration, frank erythema (plaques, bands, or diffuse), frankly swollen mucosa, aphthoid ulceration, superficial or shallow ulceration, deep ulceration, non-ulcerated stenosis, and ulcerated stenosis. The percentage of the segmental surfaces involving the disease and ulcerations are positioned on a 10-cm

analogue scale between 0 and 10 (no lesion=0, lesions or ulcerations involving 100% of the segment =10). For the ileum and for colonic segments only partially explored, the 10-cm scale represented the area actually seen (Mary *et al* 1989). The CDEIS can potentially range between 0 and 44, with higher scores reflecting greater endoscopic activity. The CDEIS correlates poorly with clinical activity (Modigliani *et al* 1990, Cellier *et al* 1994). It is fairly time-consuming, and elaboration of the score requires analogue scale transformation, making the CDEIS unsuitable for everyday clinical practice.

Table 3. Scoring system for Crohn's disease index of severity (CDEIS) (Mary *et al* 1989).

		total
Deep ulcerations (if present, score =12)	ileum, right colon, transverse colon, sigmoid and left colon, rectum	total 1
Superficial ulcerations (if present, score =6)	ileum, right colon, transverse colon, sigmoid and left colon, rectum	total 2
Surface affected by disease (cm)	ileum, right colon, transverse colon, sigmoid and left colon, rectum	total 3
Surface affected by ulcerations (cm)	ileum, right colon, transverse colon, sigmoid and left colon, rectum	total 4
	total 1+2+3+4=	total A
	number of segments totally or partially explored	*n*
	total A/*n*=	total B
	if a non-ulcerated stenosis present, add 3=	C
	if an ulcerated stenosis present, add 3=	D
	total B+C+D=	CDEIS

5.2.2 Simple endoscopic score for Crohn's disease (SES-CD)

To simplify endoscopic assessment, a simple endoscopic score for Crohn's disease (SES-CD) has been developed and validated (Daperno *et al* 2004). It correlates closely with the CDEIS and is easier and faster to score and calculate. The SES-CD is based on four variables scored in the same five ileocolonic segments as in the CDEIS (Table 4). The ileum is scored for the full extent to which it is examined. The ileal score excludes the ileocecal valve or ileocolonic anastomosis. The right colon includes the ileocecal valve, the cecum, and the ascending colon up to the hepatic flexure. The transverse colon is defined as the segment between the hepatic and splenic flexures. The left colon includes the descending colon and sigmoid. The rectum is defined as the portion distal to the

rectosigmoid junction. The score can range from 0 to 60. In the validation study, correlations of the SES-CD with serum CRP and the CDAI were significant (Daperno *et al* 2004). A modification of this score ranging from 0 to 15 served for a subgroup of patients in a "topdown" therapy study showing at week 104 a significantly lower endoscopic score in patients with early combined therapy than in those receiving conventional treatment (D'Haens *et al* 2008b).

Table 4. Definitions of the simple endoscopic score for Crohn's disease (SES-CD) (Daperno *et al* 2004).

Variable	*Score =0*	*Score =1*	*Score =2*	*Score =3*
size of ulcers	none	aphthous ulcers (diameter: 0.1 to 0.5 cm)	large ulcers (diameter: 0.5 to 2 cm)	ulcers >2cm
ulcerated surface	none	<10%	10-30%	>30%
affected surface	unaffected	<50%	50-75%	>75%
presence of narrowings	none	single, can be passed	multiple, can be passed	cannot be passed

5.2.3 Rutgeerts' score

After a curative resection of CD, endoscopic examination reveals signs of endoscopic activity in up to 60 to 70% of patients at 6 to 12 months, and severity of the lesions predicts subsequent clinical course (Rutgeerts *et al* 1984, 1990). For scoring of the disease in the ileum, Rutgeerts and coworkers (1990) have developed a score considered the gold standard for endoscopical post-surgical recurrence evaluation (Sostegni *et al* 2003). Findings in the ileum are scored in five categories: i0 is scored when no lesions occur in the distal ileum, i1 means ≤5 aphthous lesions, i2 means >5 aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions or lesions restricted to ileocolonic anastomosis, i3 represents aphthous ileitis with diffusely inflamed mucosa, and i4 means diffuse inflammation with large ulcers, nodules, or narrowing (Rutgeerts *et al* 1990).

5.3 Histologic activity

Data available on the histology and activity of CD are limited, and among expert clinicians no general agreement exists as to the use of microscopy in assessment of CD activity (Stange *et al* 2006). Due to its uncertain clinical relevance, the histologic disease activity assessment cannot in general be recommended as a treatment endpoint (Sandborn *et al* 2002). If biopsies are used, multiple samples are necessary for analysis. Several scoring systems for histologic findings exist. A scoring system for histologic findings demonstrates histologic healing in patients treated with azathioprine or infliximab (D'Haens *et al* 1997, 1998, 1999b, Geboes *et al* 2005). Epithelial damage is classified as normal (score =0), focal (score =1), or extensive (score =2) pathology; architectural changes as normal (score =0), moderately (<50%) disturbed (score =1), or severely (>50%) disturbed (score =2); infiltration of mononuclear cells in the lamina propria as a normal (score =0), moderate (score =1), or severe (score =2) increase; infiltration of polymorphonuclear cells in the lamina propria as normal (score =0), moderate (score =1), or severe (score =2) increase. Polymorphonuclear cells in the epithelium are scored 1 if in the surface epithelium, 2 if cryptitis is present, and 3 if a crypt abscess is present. When present, erosions or ulcers are scored as 1, and the presence of granuloma is scored as "yes" (score =1) or "no" (score =0). Finally, the number of biopsy specimens affected is scored none (score =0), <33% (score =1), 33% to 66% (score =2), or >66% (score =3). This scoring system is suggested for histologic CD findings in clinical trials (Sandborn *et al* 2002).

5.4 Blood tests

Peripheral blood inflammatory markers such as blood count, ESR, CRP, orosomucoid, and albumin have been routinely used for assessment of disease activity. Also several other acute-phase proteins, many cytokines, and serological markers have been studied for detection of active disease. These markers, however, reflect degree of inflammation in the whole body and are unable to detect sufficiently reliably inflammation in the GI tract. These tests thus cannot replace the need for clinical and endoscopic assessment of disease activity.

5.4.1 Blood count

In IBD, anemia, leukocytosis, and thrombocytosis are common findings. They are nonspecific, and the reason for anemia in CD can be multifactorial (iron deficiency due to blood loss, inflammation). An elevated leukocyte count may be an indicative of extensive active inflammation, but can also be a result of corticosteroid treatment. Immunosuppresive treatment may lower leukocyte count. Elevated platelet count is a nonspecific response to inflammation and may occur reactively during hemorrhage. It correlates weakly with clinical and endoscopic acitivity in CD (Cellier *et al* 1994).

5.4.2 Erythrocyte sedimentation rate

ESR indirectly measures acute-phase plasma protein concentrations. It is influenced by erythrocyte morphology and number as well as by plasma components (for example, immunoglobulins). It is not rapidly responsive to changes in clinical status. In CD, ESR appears to rise with increasing activity in colonic disease, but fails to reflect disease activity of the small bowel (Sachar *et al* 1990, Desai *et al* 2007).

5.4.3 C-reactive protein

CRP is produced predominantly in the liver in response to stimulation by IL-6 and also by TNFα, and IL-1β (Florin *et al* 2006). In the presence of an acute-phase stimulus such as inflammation, production of CRP is rapidly upregulated. The half-life of CRP is short (19 hours) and thus, when the acute phase stimulus has disappeared, CRP concentration quickly falls (Desai *et al* 2007). In addition to active gut inflammation, elevated CRP can in CD be a sign of complications such as abscesses. Whereas CD is associated with a strong CRP response, the UC response is only modest or absent (Saverymuttu *et al* 1986a, Vermeire *et al* 2006). In diagnosis of CD, CRP is helpful, and its sensitivity values range between 70% and 100% depending also on the cutoff values used (Vermeire *et al* 2006). Lower sensitivity values have been reported by Tibble and coworkers (2002), who showed CRP (>5 mg/l) sensitivity (50%) and specificity (81%) in discriminating organic bowel disease from functional symptoms. In CD, elevated CRP is associated with clinically, endoscopically, and histologically active disease (Solem *et al* 2005). During CD clinical remission, a persistently raised CRP may be associated with an earlier clinical relapse (Boirivant *et al* 1988). In clinically active CD, a low CRP value seems to be associated with ileal disease (Florin *et al* 2006).

5.4.4 Orosomucoid

The acute-phase reactant orosomucoid correlates with clinical disease activity, but its fairly long half-life (five days) makes it a less useful marker for clinical practice (Cellier *et al* 1994, Vermeire *et al* 2006).

5.4.5 Albumin

In active IBD, serum albumin often declines and correlates inversely in CD with clinical and endoscopic activity (Modigliani *et al* 1990, Cellier *et al* 1994, Solem *et al* 2005). Hypoalbuminemia may be a consequence of protein loss from the inflamed gut or may result from malnutrition secondary to inadequate protein intake or malabsorption.

5.4.6 Cytokines

TNFα is produced primarily by activated macrophages and monocytes. Although in active IBD serum TNFα is often increased, the serum concentrations are not consistently elevated and are thus of limited value as surrogate markers of IBD activity (Desai *et al* 2007).
IL-6, a cytokine produced by macrophages and T cells, has both anti-inflammatory and proinflammatory properties. In CD, IL-6 is released in increased amounts from affected mucosa, and the level correlates with endoscopic findings (Reimund *et al* 1996). Serum IL-6 concentrations correlate closely with the CDAI and are higher in those with inflammatory CD than in those with stricturing disease. Furthermore, during steroid-treatment, serum IL-6 levels decrease in parallel with clinical improvement (Reinisch *et al* 1999).

In addition, several other serum cytokines and cytokine receptors such as IL-1β, IL-2, IL-2R, IFN-γ, IL-8, IL-23, IL-27, and IL-15 have been elevated in IBD, but these seem to have limited utility as non-invasive markers of disease activity, because serum levels often remain within normal limits despite active IBD (Desai *et al* 2007).

5.4.7 Antibody responses to auto- and microbial antigens

The serologic immune markers anti-*Saccharomyces cerevisiae* antibodies (ASCA) and the anti-neutrophil cytoplasmic antibody with a perinuclear staining pattern (pANCA) increasingly serve for IBD diagnosis. ASCA is directed to the phosphopeptidomannan wall component of the common baker's yeast *Saccharomyces cerevisiae.* Elevated ASCA titers have been described in 50 to 80% of CD patients (in UC, 2 to 14%), whereas pANCA positivity is detectable in 40 to 80% of UC patients (Reese *et al* 2006, Papp *et al* 2007). These rarely appear in healthy controls. According to a meta-analysis comprising 4019 CD patients, a positive ASCA with a negative pANCA test gave a sensitivity of 55% and a specificity of 93% for CD. In pediatric patients, the sensitivity was somewhat better (70%, with a specificity of 93%) (Reese *et al* 2006). In CD, pANCA is often associated with UC-like disease (Targan *et al* 2005).

Antibody responses toward *Eschericia coli* outer membrane porin C (anti-ompC), a CD-related bacterial sequence from *Pseudomonas fluorescens* (anti-I2), and toward a flagellin CBir1 (anti-CBir1) are associated with CD in approximately 50% of patients. These markers are uncommon in UC or in a non-IBD population. Anti-I2 seems to associate with fibrostenosing small bowel CD and risk for small bowel surgery (Mow *et al* 2004). Anti-ompC and anti-CBir1 are associated with small-bowel, fibrostenosis, and intestinal-perforating disease features and anti-OmpC also with small bowel surgery (Mow *et al* 2004, Targan *et al* 2005). Patients with a triple positivity for ASCA, anti-ompC, and anti-I2 appear eight times more likely to require small bowel surgery than are seronegative patients (Mow *et al* 2004). These antibody responses may be helpful in subtyping Crohn's disease patients and in prediction of disease course or in differentiation of colitis unclassified. In monitoring of CD activity these markers are of litte value: ASCA is fairly constant over time, and antibody responses to these microbial agents (ASCA, pANCA,

anti-I2, and anti-ompC) seem to remain unchanged in the majority of CD patients during anti-TNFα therapy (Landers *et al* 2002, Papp *et al* 2007).

5.5 Radiolabeled neutrophils

White cell scans with radiolabeled leukocytes or granulocytes can be useful in detecting acute inflammation. The technique of ^{111}Indium-labeled granulocytes—rather than mixed leucocytes—has been modified for gastroenterological use. This technique involves abdominal scintigraphy and 4-day fecal collection of ^{111}Indium-labeled granulocytes. Fecal excretion of ^{111}Indium-labeled granulocytes has been considered a specific, quantitative, and sensitive measurement of intestinal inflammation (Saverymuttu 1985, Saverymuttu *et al* 1986b) and is suggested as the gold standard for assessing intestinal inflammation in CD (Gaya and Mackenzie 2002). In quiescent CD, this technique may reveal subclinical inflammation (Saverymuttu 1986). The main drawbacks of this procedure are a need for special labeling facilities, its cost, and a radiation burden.

5.6 Intestinal permeability tests

Intestinal permeability is assessed noninvasively by measuring urinary secretion of orally administered test substances. Permeability tests are non-specific for IBD. Usually two test substances based on different urinary excretion principles are combined, providing a specific index of intestinal permeability. The most often-used combination is a disaccharide (for example lactulose) and a monosaccharide (L-rhamnose or mannitol). Many other substances used include ^{51}Chromium-ethylene diaminetetraacetic acid (^{51}Cr-EDTA), ^{99}Technetium-diethylene triaminepentaasectic acid (^{99}Tc-DPTA), polyethylene glycol (PEG) (Bjarnason *et al* 1995), and even the water-soluble contrast medium iohexol (Halme *et al* 2000). Intestinal permeability tests are more suitable to assessment of small bowel disease activity than that of colonic CD. These are sensitive for detection of active small bowel CD and are considered useful tools for disease activity assessment (Bjarnason *et al* 1995). Lactulose-mannitol and iohexol tests have correlated with endoscopic and clinical CD activity (Halme *et al* 2000). In clinical CD remission, an abnormal permeability test seems to predict clinical relapse (Wyatt *et al* 1993, Tibble *et al* 2000b, Arnott *et al* 2000). Fecal and gut lavage fluid calprotectin levels correlate with intestinal permeability tests in CD (Berstad *et al* 2000, Tibble *et al* 2002), and an abnormal calprotectin in combination with increased intestinal permeability gives an odds ratio of 15.0 for small bowel disease (Tibble *et al* 2002). However, diagnostic accuracy in distinguishing between IBD and non-IBD conditions with an intestinal permeability test is inferior to use of fecal calprotectin (Canani *et al* 2006).

6 Calprotectin

6.1 Structure and functions of calprotectin

Called provisionally L1 or leukocyte-derived L1 protein, calprotectin was isolated from human granulocytes first by Fagerhol and coworkers (1980). It is a calcium- and zinc-binding protein complex consisting of two heavy (14kDa) polypeptide chains and one light (8kDa) chain and has a molecular mass of 36.5 kDa (Dale *et al* 1983). The subunits of calprotectin have also been termed L1 light and heavy chains (Fagerhol *et al* 1980), migration inhibitory factor-related protein 8 (MRP8) and MRP14 (Odink *et al* 1987), calgranulin A and B (Wilkinson *et al* 1988), and S100A8 and S100A9 (Schafer *et al* 1995). The light chain is identical to the cystic fibrosis-associated antigen (CFAg) decribed in 1973 (Wilson *et al* 1973). Calprotectin is a member of the S-100 protein family. Phagocytic S100 proteins are among damage-associated molecular pattern proteins (DAMPs) that are endogenous molecules released by activated or damaged cells under conditions of cell stress (Foell *et al* 2008). Genes coding for calprotectin are mapped to chromosome 1q21r (Schafer *et al* 1995). Calprotectin is a major component of human neutrophil granulocytes, constituting about 5% of their total protein and up to 60% of their cytosol protein (Fagerhol *et al* 1980, Dale *et al* 1985). Smaller amounts exist in the plasma membrane and nucleus. Calprotectin is also expressed in activated macrophages and monocytes, but is undetectable in lymphocytes (Dale *et al* 1985, Johne *et al* 1997).

The biological functions of calprotectin are not fully known. Upon neutrophil activation or upon endothelial adhesion of monocytes, calprotectin is released and is detectable in serum, body fluids, and feces. DAMPs promote inflammation through activation of PRRs: Calprotectin activates macrophages via interaction with TLR4 (Vogl *et al* 2007). Antimicrobial and fungistatic properties are mediated through the zinc-binding capacity of calprotectin (Steinbakk *et al* 1990). Calprotectin can inhibit zinc-dependent matrix metalloproteinases needed in many biological functions such as in wound healing, inflammation, angiogenesis, and tissue destruction (Střiž *et al* 2004). Calprotectin has also apoptosis-inducing properties, immunoregulatory functions, and cytotoxic and growth-inhibitory effects (Yui *et al* 1997, 2003, Johne *et al* 1997, Mikami *et al* 1998). Excessive concentrations of calprotectin may induce cell and tissue damage (Yui *et al* 2003).

6.2 Plasma calprotectin

Calprotectin can be measured in plasma (Berntzen *et al* 1991), synovial fluid (Berntzen *et al* 1991), saliva (Cuida *et al* 1995), cerebrospinal fluid (Dunlop *et al* 1991), and urine (Holt *et al* 1983). It is absent from healthy skin, but is expressed in various inflammatory dermatoses (Brandtzaeg *et al* 1992). Plasma calprotectin levels are about 30% higher in men than in women (Dale 1990). Its serum or plasma levels are increased in many diseases in which neutrophils are recruited, diseases such as rheumatoid arthritis (Berntzen *et al* 1991), polymyalgia rheumatica (Brun *et al* 2005, Korndorfer *et al* 2007), cystic fibrosis

(Golden *et al* 1996), chronic bronchitis (Stockley *et al* 1984, Roth *et al* 1992), acute allograft rejection (Burkhardt *et al* 2001), gut inflammation (Leach *et al* 2007), alcohol-induced liver cirrhosis (Homann *et al* 1995), acute pancreatitis (Carroccio *et al* 2006), preeclampsia (Braekke *et al* 2005), some malignant diseases (Kristinsson *et al* 1998, Odegaard *et al* 2008), and active multiple sclerosis (Bogumil *et al* 1998). In severe bacterial infections, plasma calprotectin levels may increase to 40- to 130-fold, whereas in viral infections, concentration may be normal or only slightly elevated (Sander *et al* 1984).

6.3 Fecal calprotectin

6.3.1 Fecal calprotectin assay

Using polyclonal rabbit calprotectin antibodies, Roseth and coworkers (1992) developed a method for extraction of calprotectin from feces and quantification by an enzyme-linked immunoassay (ELISA). In 33 healthy volunteers, the median fecal calprotectin was 2 mg/l (milligram calprotectin per liter of fecal homogenate) and in the non-IBD control group 10 mg/l, both of which were significantly lower than the fecal calprotectin concentrations in their CD patients (n=21, 43 mg/l) or in UC patients (n=17, 40 mg/l) (Roseth *et al* 1992). In the presence of Ca^{2+}, calprotectin is resistant to degradation and at room temperature stable in stool for up to seven days (Roseth *et al* 1992). This makes possible transport of samples to the laboratory by ordinary mail. Fecal calprotectin levels in healthy individuals are approximately six times as high as in plasma (Angriman *et al* 2007).

An improved immunoassay for fecal calprotectin was published in 2000 (Ton *et al* 2000). The increased calprotectin yield with this method resulted from dissociating agents in the extraction solution and an increased ratio between extraction solution and feces. Compared to the older method, the separation between normal and pathological values is better with the improved method and an approximately five-fold increase in fecal calprotectin concentration occurs. Both the average daily fecal calprotectin excretion and the calprotectin concentration of a single spot-sample correlate closely with fecal ^{111}Indium-labelled granulocyte excretion (Roseth *et al* 1999, Tibble *et al* 2000a). Need for only 50 to 100 mg feces in the new method, compared to 5 g in the original method, makes sample collection more convenient for the patient. The normal level of fecal calprotectin is suggested to be <50 µg/g. However, using meta-analytical techniques, von Roon *et al* (2007) found higher precision at a cutoff of 100 µg/g in distinguishing IBD from non-IBD conditions. In inflammation, calprotectin concentration in feces can rise as high as to tens of thousands µg/g (Fagerhol 2000). The concentrations measured with the older assay can be converted to the newer assay by multiplying them by five (Ton *et al* 2000). A polyclonal calprotectin ELISA test is commercially available (PhiCal Test®, Calpro AS, Oslo, Norway and Calprest®, Eurospital, Trieste, Italy). Furthermore, a monoclonal calprotectin test is available (Bühlmann Laboratories AG, Schönenbuch, Switzerland) and an assay using two selected monoclonal antibodies (Immundiagnostik AG, Bensheim, Germany) is used in some studies (Langhorst *et al* 2005, 2008, Schröder *et al* 2007).

Recently, rapid semi-quantitative tests have been developed and validated for identifying patients with GI inflammation (Damms *et al* 2008, Vestergaard *et al* 2008, Otten *et al* 2008). A quantitative ELISA test seems, however, to be more suitable for monitoring of IBD activity than these rapid tests.

6.3.2 Fecal calprotectin excretion

Calprotectin excretion may reflect increased neutrophil and mononuclear cell migration into the gut lumen through the inflamed mucosa (Roseth *et al* 1999). Fecal calprotectin is specific for inflammation, but not disease-specific, as it may, besides IBD, be elevated also in inflammatory conditions such as enteropathy caused by non-steroidal anti-inflammatory drugs (NSAIDs) (Tibble *et al* 1999), in gastrointestinal neoplasms (Roseth *et al* 1993, Kristinsson *et al* 1998, Tibble *et al* 2001, Johne *et al* 2001, Hoff *et al* 2004), gastrointestinal infections (Hoff *et al* 2004, Kristinsson *et al* 1998), necrotising enterocolitis (Carroll *et al* 2003), food allergies (Carrocio *et al* 2003), acute radiation-induced proctitis (Larsen *et al* 2004, Hille *et al* 2008), and intestinal allograft rejection (Sudan *et al* 2007). Calprotectin concentrations may also be elevated in subjects with microscopic colitis (Limburg *et al* 2000) and in patients with pouchitis (Thomas *et al* 2000). In untreated celiac disease, in which the mucosal inflammation is typically lymphocyte-predominant, the fecal calprotectin concentration does not necessary differ from that of controls (Montalto *et al* 2007a). Furthermore, in patients with liver cirrhosis, fecal calprotectin can be falsely positive, possibly due to mucosal abnormalities in patients with portal hypertension (Carrocio *et al* 2003).

Fecal calprotectin is already present in meconium even at low gestational ages (Laforgia *et al* 2003), and it is—apparently as a normal phenomenon— higher (>10-fold) in infants in the first year of life than in children over one year (Olafsdottir *et al* 2002, Campeotto *et al* 2004). From that age up to 17-year-old healthy children, median calprotectin concentration seems to be similar to that of healthy adults (Carroccio *et al* 2003, Fagerberg *et al* 2003, Baldassarre *et al* 2007). Interestingly, in adults, stool calprotectin seems to increase with age and has a significant positive relationship to obesity and physical inactivity and an inverse relationship to fiber intake and vegetable consumption—all of these lifestyle factors being associated with colorectal cancer (Poullis *et al* 2004). Probably representing subclinical inflammation, elevated calprotectin concentrations occur in relatives of CD and UC patients and also in relatives of ankylosing spondylitis patients (Thjodleifsson *et al* 2003, Bjarnason *et al* 2003, Montalto *et al* 2007b).

A day-to-day variation in calprotectin excretion during three consecutive days has been demonstrated both in IBD patients and in controls (Roseth *et al* 1999). A similar biological day-to-day variation in fecal calprotectin among 14 patients scheduled for colonoscopy without colonic neoplasm or inflammation has occurred in 64% of patients, whereas values were noticeably stable in the remaining 36% (Husebye *et al* 2001). A high number of replicates (n=8) revealed this feature, and 20% of the measurements exceeded 50 µg/g. The reason for this bimodal pattern of calprotectin excretion is unknown, but it must be considered in any interpretation of a sole stool test in clinical practice (Husebye *et al*

2001). Preparation of the bowel for colonoscopy does not affect the stool calprotectin levels: In 17 patients, pre-and post-colonoscopy levels did not differ significantly (Summerton *et al* 2002).

6.3.3 Fecal calprotectin for diagnosis of IBD

The first studies showing the value of calprotectin in distinguishing organic intestinal disease from functional disorders were published in 2000. Tibble *et al* (2000a) studied 220 consecutive adult patients with bowel symptoms suggestive of CD or IBS and found organic disease in 28%. Using a cutoff of 10 mg/l, the sensitivity and specificity of fecal calprotectin in discriminating between organic and functional intestinal disease was 82% and 83% (Tibble *et al* 2000a, Konikoff and Denson 2006). With a raised cutoff of 30 mg/l, the sensitivity was 100% and specificity 97% in distinguishing between active CD and IBS (Tibble *et al* 2000a, Gisbert and McNicholl 2008). Later, a larger study of Tibble's group measured fecal calprotectin in 602 consecutive patients with abdominal symptoms (Tibble *et al* 2002). All patients provided stool samples for measurements of calprotectin, underwent an intestinal permeability test and radiological or endoscopic examinations for diagnosis; their symptoms were classified according to clinical criteria for IBS, Rome I criteria. Of these 602 patients, 263 (44%) had an organic bowel disease and 339 (56%) functional. At a fecal calprotectin cutoff level of 10 mg/l, the sensitivity was 89% and specificity was 79% for organic bowel disease.

Limburg *et al* (2000) studied 110 adult patients referred for colonoscopy for chronic diarrhea or for chronic colitis of unknown activity. Increased calprotectin levels were significantly associated with colorectal inflammation, whereas fecal hemoglobin levels were not. At a cutoff level of 100 µg/g, the sensitivity for any colorectal inflammation was 83% and the specificity 83%. The specificity rose to 90% when only those with histologically confirmed normal colorectal mucosa were considered, and when only IBD patients were considered, the sensitivity increased to 94% (Limburg *et al* 2000). In further studies, the sensitivity in adults has ranged from 63% to 100% and the specificity from 80 to 100% in differentiating IBD from functional disorders. (Carroccio *et al* 2003, Costa *et al* 2003, Dolwani *et al* 2004, Konikoff and Denson 2006, D'Inca *et al* 2007, Schoepfer *et al* 2007, 2008, Kaiser *et al* 2007, Schröder *et al* 2007). Accordingly, positive predictive value (PPV) has been between 70 and 100% and negative predictive value (NPV) between 51 and 91% (Carroccio *et al* 2003, Costa *et al* 2003, Konikoff and Denson 2006, D'Inca *et al* 2007, Schoepfer *et al* 2007, 2008, Kaiser *et al* 2007, Schröder *et al* 2007).

A meta-analysis based on prospective studies has evaluated the diagnostic precision of fecal calprotectin for IBD (von Roon *et al* 2007). A control group comprised patients with IBS or healthy controls. A total of 663 patients (both adults and children) had CD, 361 UC, and 183 patients were considered to have IBD. Fecal calprotectin concentrations in IBD patients were higher by 219 µg/g than in healthy controls. In CD patients, calprotectin levels were higher than in IBS patients by 304 µg/g. For diagnosis of IBD, a sensitivity of 89% and a specificity of 81% was calculated from the data of nine studies (Tibble *et al*

2000a, 2001, Bunn *et al* 2001a, Summerton *et al* 2002, Fagerberg *et al* 2005, Bremner *et al* 2005, Thjodleifsson *et al* 2003, Costa *et al* 2003, Carroccio *et al* 2003).

Silberer *et al* (2005) studied fecal calprotectin, lactoferrin, polymorphonuclear neutrophil elastase (PMN-E), lysozyme, and alpha1-antitrypsin (α1-AT) in patients referred to colonoscopy and found PMN-E and calprotectin to be better than other markers in differentiation of IBD from IBS. Compared to calprotectin, however, the granulocyte-specific protein S100A12 seemed to show even better sensitivity and specificity in distinguishing active IBD from IBS (Kaiser *et al* 2007).

For the diagnosis of IBD, fecal calprotectin appears to be superior to serological markers such as CRP, ESR, ANCA, or ASCA. In several studies, the sensitivity of CRP has ranged from 35 to 64% and the specificity 78 to 100% (Tibble *et al* 2000a, Canani *et al* 2006, Silberer *et al* 2005, Fagerberg *et al* 2003, Schoepfer *et al* 2008). Accordingly, for ESR, sensitivity ranged from 18 to 52% and specificity from 78 to 100% (Tibble *et al* 2000, Canani *et al* 2006, Silberer *et al* 2005, Fagerberg *et al* 2003). Overall diagnostic accuracy for CRP (65%) has been inferior to that of calprotectin (81%) or lactoferrin (77%) (Langhorst *et al* 2008). Furthermore, in predicting abnormal small bowel radiology, calprotectin seems to be more sensitive (100%) and specific (79%) than is a combination of elevated CRP and ESR (sensitivity 50%, specificity 84%) (Dolwani *et al* 2004). In CD patients after ileocolonic resection, CRP correlated significantly with both fecal calprotectin and lactoferrin ($r=0.53$, $p<0.01$), but ESR correlated weakly only with calprotectin ($r=0.28$, $p=0.03$) (Scarpa *et al* 2007).

In Schoepfer and coworkers' study (2008) comparing calprotectin, lactoferrin, CRP, and serological CD markers (ASCA + and pANCA + or -) in distinguishing CD from IBS, the sensitivity, specificity, PPV, and NPV of CD markers were 63%, 96%, 95%, and 69%, while these values for calprotectin were 83%, 100%, 100%, and 83% and for lactoferrin 83%, 96%, 96%, and 83%. Only marginal diagnostic accuracy resulted from combining calprotectin or lactoferrin with serological markers. In a recent Finnish pediatric study, however, combination of fecal calprotectin with ASCA, anti-I2, and with antibodies to *Bacteroides caccae* TonB-linked outer membrane protein (OmpW) resulted in a sensitivity of 100%, specificity of 36%, PPV of 66%, and NPV of 100% in detecting CD (Ashorn *et al* 2008).

6.3.4 Fecal calprotectin and disease activity in CD

Fecal calprotectin concentrations are elevated in both adults (Table 5) and children (Bremner *et al* 2005, Bunn *et al* 2001a, 2001b, Canani *et al* 2006, 2008) with IBD. In CD, the CDAI is widely used as a measure of disease activity, but it correlates fairly weakly with fecal calprotectin (Costa *et al* 2003, Gaya *et al* 2005, Denis *et al* 2007, Scarpa *et al* 2007). Calprotectin is often elevated also in clinical remission: in a study by Costa and coworkers (2003), patients with the CDAI >150 had a median calprotectin 405 μg/g whereas during clinical remission it was 213 μg/g. Furthermore, in one study of Tibble and coworkers (2000a), CD patients with clinically active disease (assessed with HBI) had a median calprotectin of 165 mg/l (converted to a newer assay, 825 μg/g) and in clinical

remission 87 mg/l (newer assay: 435 μg/g). In children with HBI-assessed clinical remission, median fecal calprotectin was 1293 μg/g, whereas in clinically active disease it was 2557 μg/g (Walkiewicz *et al* 2008). In another study, patients in clinical remission (41 of 49 patients with a CDAI ≤150) had an elevated calprotectin concentration 47 mg/l (converted to newer assay: 235 μg/g) (Thjodleifsson *et al* 2003). In 11 patients with a clinical relapse (CDAI >151) after an ileocolonic resection, median fecal calprotectin level was 233 μg/g, while in those 46 patients in clinical remission it was 217 μg/g (p=0.968) and correlation of the CDAI with calprotectin was nonsignificant (Pearson´s r=0.13, p=0.34) (Scarpa *et al* 2007). It seems that this relatively poor correlation of the CDAI with fecal calprotectin is due to the fact that CDAI is a clinical score and is insufficiently sensitive to detect intestinal inflammation.

A close correlation of the fecal ^{111}Indium-labeled granulocyte-excretion—technique suggested as a gold standard for assessing intestinal inflammation (Gaya and Mackenzie 2002)—with fecal calprotectin occurs in UC and CD (Roseth *et al* 1999, Tibble *et al* 2000a). In 35 clinically active CD patients, calprotectin gave a sensitivity of 80%, a specificity of 67%, a PPV of 87%, and a NPV of 60% in detecting those CD patients who had inflammation in a white-cell scan (Gaya *et al* 2005).

Calprotectin correlates significantly with endoscopic and histologic activity in UC (Roseth *et al* 1997) and in pediatric IBD patients (Bunn *et al* 2001b), and its normal level in clinical IBD remission correlates closely with endoscopic mucosal healing both in children and in adults (Bunn *et al* 2001b, Roseth *et al* 2004, Fagerberg *et al* 2007). In CD, as well, significant correlations between fecal calprotectin concentrations and endoscopic disease activity have appeared (Silberer *et al* 2005, D'Inca *et al* 2007, Langhorst *et al* 2008, Schoepfer *et al* 2008), although these correlations may not be as apparent as in UC. In D'Inca and coworkers' study (2007), endoscopic CD findings correlated with calprotectin, but not with histologic score. Lactoferrin, failed, however, to correlate with endoscopic score, but correlated with histology (D'Inca *et al* 2007). In Silberer *et al*'s study (2005), calprotectin and PMN-E were superior to lactoferrin, lysozyme, and α1-AT in detecting differences between endoscopically assessed IBD activity groups. When endoscopic inflammation in CD was present, calprotectin levels were elevated compared to findings with no inflammation (105 μg/g and 11 μg/g, p<0.01) (Langhorst *et al* 2008). Moreover, when the cutoff point SES-CD <20 was set for endoscopically mildly active CD, calprotectin was significantly higher in patients with moderately or severely active disease than in those with mild disease (mean calprotectin ± standard deviation SD 433.0±42.6 μg/g versus 112.1±31.3, p<0.001) (Schoepfer *et al* 2008). Interestingly, in Kaiser and coworkers' study (2007), differences in calprotectin values between patients with active (median±95% confidence interval CI was 97±45 μg/g) and inactive IBD (19±158 μg/g) failed to reach statistical significance. In that study, IBD was considered inactive, when no signs of endoscopic and histologic inflammation were present and clinical activity scores were normal.

In children with inflammatory bowel disease (26 CD, 32 UC), fecal calprotectin has shown a good correlation with histologic grade of mucosal inflammation (r=0.655), and this correlation was similar to that observed for endoscopy (r=0.699) (Canani *et al* 2008). The sensitivity and specificity of fecal calprotectin in detecting histolocially active mucosal

inflammation was 94% and 64%. Accordingly, for endoscopy, the sensitivity as 67% and specificity was 82% (Canani *et al* 2008).

No correlation appeared between the CDEIS and calprotectin or any other clinical or biological markers (ESR, acid α-1 glycoprotein, IL-6, IL-8, TNF-receptor type 2, and soluble IL-2R) in 28 CD patients with clinically active disease (CDAI >150) and normal CRP levels (Denis *et al* 2007).

Studies of calprotectin in monitoring treatment in CD are scarce. Following infliximab therapy, a transient decrease in fecal calprotectin concentrations in two CD patients corresponded to an improvement in clinical disease activity, but no endoscopic activity evaluation was performed (Aadland and Fagerhol 2002). In pediatric IBD patients during glucocorticoid therapy, fecal calprotectin decreased in line with clinical improvement, but seldom fell within the normal range–suggesting ongoing inflammation despite clinically quiescent disease (Kolho *et al* 2006). Exploration of repeated fecal calprotectin, lactoferrin, PMN-E, and lysozyme concentrations in 31 UC patients showed significantly higher calprotectin, lactoferrin, and PMN-E (but not lysozyme) values in the active disease than in the inactive disease phase (Langhorst *et al* 2005).

Table 5. Fecal calprotectin in adults with IBD. Modified from Konikoff MR, Denson LA. Inflamm Bowel Dis 2006;12:524-533.

reference	*patients n, disease*	*assessment of activity*	*calprotectin active disease, µg/g*	*calprotectin inactive disease, µg/g*	*calprotectin controls or IBS µg/g*
Roseth *et al* 1992	21 CD	clinical	218*	-	10*
	17 UC		98*	-	
Roseth *et al* 1997	62 UC	endoscopy	325*	70 *	30*
		histology	340*	60*	
Roseth *et al* 1999	19 CD	clinical remission	-	496*	58*
	10 UC	clinical remission	-	752*	
Roseth *et al* 2004	17 CD	clinical and endoscopic	-	35	-
	28 UC	(44/45) remission	-	16	
Limburg *et al* 2000	16 UC or CD	endoscopy histology	1722	-	31
Tibble *et al* 2000b	43 CD	CDAI <150, HBI <4	-	475*	-
	37 UC	HBI <4	-	290*	-
Tibble *et al* 2000a	116 CD	HBI cutoff 5	825*	435*	10*
Tibble *et al* 2000a	31 CD	symptoms, diagnostic procedures	675*	-	10*
Summerton *et al* 2002	4 CD	endoscopy	164*	-	23*
	10 UC		267*	-	
Costa *et al* 2003	49 CD	CDAI cutoff 150	405	213	22
	82 UC	UCAI cutoff 4	327	123	
Carrocio *et al* 2003	10 CD	CDAI >150 in 8/10	320	-	20
Thjodleifsson *et al* 2003	49 CD	CDAI ≤150 in 41/49	-	235*	20*
Dolwani *et al* 2004	25 CD	CDAI >150 in 23/25	227	-	10
Wassel *et al* 2004	25 CD	CDAI >150	230	-	9
Costa *et al* 2005	38 CD	CDAI <150	-	220	
	41 UC	UCAI <4	-	151	-
Langhorst *et al* 2005	31 UC	CAI cutoff 5	57	0.1	-
D'Inca *et al* 2007	46 UC	endoscopy	340	161§	-
Denis *et al* 2007	28 CD	CDEIS cutoff 6	77	122	-
Schröder *et al* 2007	25 CD	CDAI >150	143	-	6
	20 UC	CAI ≥4	137	-	
Kaiser *et al* 2007	59 UC or CD	endoscopic, histologic,clinical	97	19	19
Scarpa *et al* 2007	57 CD	CDAI cutoff 150	233	217	-
Langhorst *et al* 2008	43 CD	endoscopy	105	11	7
	42 UC		109	35	
Schoepfer *et al* 2008	36 CD	endoscopy (SES-CD)	433†	112†§	19†
	28 UC	endoscopy (Rachmilevitz score)	390†	123†§	
D'Inca *et al* 2008	65 CD	CDAI <150	-	99	-
	97 UC	ET <3	-	101	-

* Converted to newer assay. Median concentrations, if not mentioned otherwise. † mean concentration. IBS, irritable bowel syndrome; UC, ulcerative colitis; CD, Crohn's disease; CAI, colitis activity index; CDAI, Crohn´s disese activity index; CDEIS, Crohn´s disease index of severity; SES-CD, simple endoscopic score for Crohn's disease; ET, Edwards and Truelove score; HBI, Harvey Bradshaw index. § mildly active disease.

6.3.5 Fecal calprotectin in estimating CD relapse

Typical for both CD and UC are mostly unpredictable activity relapses and longer or shorter remission periods. Preventive or early treatment would be possible, were an accurate marker for estimating relapse risk available. In IBD patients in clinical remission, Tibble et al (2000b) showed a single fecal calprotectin measurement to be a reliable tool in predicting clinical relapse within a year. At 50 mg/l (converted to newer assay: 250 μg/g), the sensitivity in all IBD patients was 90% and specificity was 83%. In that study, neither CRP nor ESR was useful in predicting relapse. Costa et al (2005), however, found fecal calprotectin to be more reliable for UC than for CD in estimating relapse of symptomless patients: In CD, the median calprotectin value in those who relapsed was 220 μg/g compared to 221 μg/g in non-relapsing patients ($p=0.395$), and the specificity was only 43%, whereas in UC, median calprotectin in relapsing patients was 221 μg/g and in non-relapsing patients 67 μg/g ($p<0.0001$). A fecal calprotectin concentration exceeding 150 μg/g showed a 2-fold risk for relapse for CD and a 14-fold relapse risk for UC. Recently, fecal calprotectin proved to be useful in predicting relapse risk in UC, but in CD did so only for a subgroup of patients with colonic CD (D'Inca *et al* 2008). In children, fecal calprotectin level >400 μg/g seemed to predict relapse risk in a 9-month follow-up: 90% of relapsing CD patients had a calprotectin concentration over this limit (Walkiewicz *et al* 2008). A retrospective analysis of pediatric IBD patients showed a calprotectin value of 275 μg/g to have a sensitivity and NPV of 97% and a specificity and PPV of 85% in predicting histologic relapse (Diamanti *et al* 2008).

7 Lactoferrin

7.1 Structure and functions of lactoferrin

Lactoferrin was first isolated from bovine milk almost 50 years ago (Groves 1960, Johanson 1960). The most striking characteristic of this 80-kDa glykoprotein was its intense red color when incubated in the presence of Fe^{2+} ions. The size and structure of lactoferrin is closely related to those of another group of iron-binding proteins, transferrins (Baker and Baker 2005, Levay and Viljoen 1995). Lactoferrin consists of a single polypeptide chain folded into two globular lobes, each having one iron-binding site (Ward *et al* 2002). Besides mammalian milk, lactoferrin is a component of many external secretions such as saliva, tears, semen, bile, and mucosal secretions (Baker and Baker 2005, Levay and Viljoen 1995). Lactoferrin is present in relatively low concentrations in plasma and is predominantly neutrophil-derived. During pregnancy, lactoferrin levels in plasma rise. Plasma lactoferrin can be elevated in iron overload, in inflammation, infectious diseases, and during tumor development (Levay and Viljoen 1995).
In polymorphonuclear neutrophils, lactoferrin is the major component of the secondary granules released by activated neutrophils during any inflammatory process. Monocytes

and lymphocytes produce no lactoferrin (Angriman *et al* 2007). Lactoferrin plays various roles in the body's host defense and in iron metabolism by controlling iron availability. It also has both bacteriostatic and bacteriocidal properties and can affect the proliferation of other microbes such as fungi and viruses. It also plays a role in cellular proliferation (Levay and Viljoen 1995, Ward *et al* 2002).

7.2 Fecal lactoferrin

Fecal lactoferrin is less studied than is calprotectin. Fecal lactoferrin is stable and easy to detect and measure in stool by ELISA (Uchida *et al* 1994, Sugi *et al* 1996, Kayazawa *et al* 2002). Also available is a qualitative latex agglutination test (Fine *et al* 1998, Schoepfer *et al* 2008) and a rapid immunocromatographic test (Otten *et al* 2008), but these are more suitable for noninvasive evaluation of organic bowel disease than for monitoring of disease activity in IBD. Like calprotectin, fecal lactoferrin is a nonspecific marker of intestinal inflammation: Besides in IBD (Sugi *et al* 1996, Kayazawa *et al* 2002), elevated concentrations have been detected in infective diarrhea (Guerrant *et al* 1992, Dai *et al* 2007), in colon cancer (Uchida *et al* 1994), and in acute radiation-induced proctitis (Larsen *et al* 2004, Hille *et al* 2008). In pouchitis, too, lactoferrin levels and clinical disease activity correlate (Parsi *et al* 2004).

An upper reference value of 2.4 µg/g has been suggested by Uchida et al (1994), whereas the upper cutoff value of the ELISA manufacturer is 7.25 µg/g (IBD-SCAN, Techlab, Blacksburg, VA, USA), and it is used in several studies (Langhorst *et al* 2005, 2008, Schoepfer *et al* 2007, 2008).

7.2.1 Fecal lactoferrin for diagnosis of IBD

In distinguishing IBD from IBS, the sensitivity of a quantitative fecal lactoferrin assay is, according to several studies, between 78 and 87% and its specificity between 85 and 100% (Kane *et al* 2003, Schoepfer *et al* 2007, 2008, D'Inca *et al* 2007, Schröder *et al* 2007). Accordingly, the PPV has been between 87% and 100% and NPV 77% and 81% (Schoepfer *et al* 2007, 2008, D'Inca *et al* 2007, Schröder *et al* 2007). In 103 patients with diarrhea of unknown origin, the qualitative latex-agglutination test gave a sensitivity of 90% and a specificity of 98% in detecting UC or CD (Fine *et al* 1998). In that study, of nine patients with microscopic colitis, eight had a negative lactoferrin test.

7.2.2 Fecal lactoferrin and disease activity in CD

Table 6 presents fecal lactoferrin concentrations in active and inactive IBD. Fecal lactoferrin levels are significantly higher in both clinically active UC and CD than in controls or in inactive disease (Sugi *et al* 1996, Kane *et al* 2003, Dai *et al* 2007). Levels are also significantly higher in endoscopically moderate or severely active CD than in mild

disease (Schoepfer *et al* 2007, 2008). When endoscopic inflammation in CD was present, lactoferrin level was significantly higher than in those with no inflammation (55.1 μg/g and 6.4 μg/g, $p<0.01$) (Langhorst *et al* 2008) A significant difference also existed between endoscopically mildly active disease (SES-CD <20) and more active disease (mean lactoferrin±SD 37.9±11 μg/g versus 221.5±22.8 μg/g, $p<0.0001$) (Schoepfer *et al* 2008). Furthermore, in florid or mild endoscopic inflammation, lactoferrin was significantly higher than in healthy controls ($p=0.0059$) or in IBD remission (Silberer *et al* 2005).
In a study by Scarpa and coworkers (2007), fecal lactoferrin failed to correlate with the CDAI in patients who had undergone a prior ileocolonic resection (Pearson´s $r=0.24$, $p=0.07$). Patients with diarrhea had significantly higher lactoferrin concentrations than did asymptomatic ones (median 25.5 μg/g versus 4.1 μg/g, $p=0.003$). This difference was not as evident with calprotectin (calprotectin in diarrhea patients 384.4 μg/g and in asymptomatic patients 263.9 μg/g, $p=0.029$).
In five children with severe CD, fecal lactoferrin concentrations decreased quickly as a consequence of infliximab therapy (Buderus *et al* 2004). Moreover, in pediatric IBD patients being weaned from steroid therapy, fecal lactoferrin was higher than in clinically inactive disease, but lower than in patients with clinically active disease but receiving no steroids (Walker *et al* 2007).

Table 6. Fecal lactoferrin levels in adult and pediatric IBD.

reference	*patients n disease*	*assessment of activity*	*Lactoferrin in active disease µg/g*	*Lactoferrin in inactive disease µg/g*	*Lactoferrin controls or IBS µg/g*
Uchida et al 1994	18 CD§ 28 UC	CDAI cutoff 150 clinical and colonoscopy	191.7† 307.4†	25.1† 63.3†	0.8†
Kayazawa et al 2002*	23 CD 27 UC	CDAI cutoff 150 colonoscopy	28.6† 37.1†	3.6† 1.3†	0.9†
Kane *et al* 2003	104 CD 80 UC	HBI cutoff 4 HBI cutoff 4	550† 2020†	204† 136†	1.3†
Dai *et al* 2007	18 CD 59 UC	HBI cutoff 4 HBI cutoff 4	1035.3† 1126.3†	133.5† 96.6†	2.5†
Schröder *et al* 2007	25 CD 20 UC	CDAI>150 CAI≥4	45 63	- -	0.2
Walker et al 2007	75 CD 60 UC	HBI cutoff 4 HBI cutoff 4	2465† 3121†	1055† 79†	1.2†
Scarpa *et al* 2007	63 CD	CDAI cutoff 150	8.3	5.5	-
Schoepfer *et al* 2008	36 CD	SES-CD cutoff 20	221.5†	37.9 † (mildly active)	2.2†
	28 UC	Rachmilevitz score cutoff 4	202.3†	124.8 † (mildly active)	
Langhorst *et al* 2008	43 CD 42 UC	colonoscopy	55.1 51.1	6.4 4.3	1.8

† mean concentration. * measurements performed on whole-gut lavage fluid. Abbreviations as in Table 5. IBD, inflammatory bowel disease. § 52 samples of CD patients and 58 samples of UC patients.

8 Other stool tests

For detection of IBD activity, this section comprises the several other stool tests studied, which presents the fecal tests most often used.

8.1 Alpha1-antirypsin

Alpha1-antitrypsin is a protease inhibitor produced by the liver, macrophages, and intestinal epithelium. It is stable in feces and can be measured by commercially available assays. It is suggested to represent fecal protein loss, but does not directly reflect intestinal inflammation (Sutherland *et al* 2008). In active CD, fecal α1-AT excretion is increased and

correlates with clinical activity (Roseth *et al* 1992, Sutherland *et al* 2008). Fecal lactoferrin, which correlates significantly with α1-AT, detects CD patients with inflammation and normal α1-AT and seems to be a more suitable marker for clinical application than is α1-AT (Sugi *et al* 1996). Sensitivity of α1-AT in distinguishing IBD from IBS is inferior to calprotectin, lactoferrin, PMN-E, myeloperoxidase (MPO), and lysozyme (Silberer *et al* 2005). However, based on a prospective study of 26 ileal CD patients, α1-AT may serve as an indicator of clinical relapse (Biancone *et al* 2003).

8.2 Polymorphonuclear neutrophil elastase

Polymorphonuclear neutrophil elastase is a neutral proteinase released from neutrophil azurophilic granules by activation. It can be measured as a complex with elastase bound to α-1-AT or as free fecal elastase (Sutherland *et al* 2008). Fecal PMN-E correlates closely with fecal lactoferrin, but its stability is inferior to that of lactoferrin (Uchida *et al* 1994, Sugi *et al* 1996). Compared to PMN-E, MPO, or lysozyme, lactoferrin seems to be a more suitable surrogate marker of inflammation (Sugi *et al* 1996, Kayazawa *et al* 2002). Furthermore, the sensitivity of PMN-E in distinguishing between IBD and IBS has been 84% and specificity 87%, with values lower than those of calprotectin or lactoferrin (Schröder *et al* 2007). Silberer and coworkers (2005), found, however, that in distinguishing between IBD and IBS or healthy controls, PMN-E and calprotectin are more accurate than MPO, lysozyme, and lactoferrin. Furthermore, PMN-E and calprotectin correlate with the endoscopically classified severity of inflammation. A study of Langhorst and coworkers (2008) showed fecal PMN-E levels to be significantly higher in active than in inactive IBD, but calprotectin's having the highest accuracy in CD.

8.3 Myeloperoxidase

Myeloperoxidase is another neutrophil product released from primary granules. Like lactoferrin, MPO is stable in whole-gut lavage fluid and correlates significantly with endoscopic findings in UC and with CD clinical activity (Kayazawa *et al* 2002). In Silberer and coworkers' report (2005), MPO seemed to separate healthy controls and IBS patients from IBD patients less effectively than did PMN-E or calprotectin.

8.4 Lysozyme

Lysozyme, known also as muramidase, is a neutrophil-derived enzyme that mediates degradation of bacterial cell walls. It occurs both in azurophilic and in secondary neutrophil granules. The excretion of lysozyme is increased in IBD, particularly in colitis (Poullis *et al* 2002, Sutherland *et al* 2008). The stability of fecal lysozyme is inferior to that of calprotectin, lactoferrin, PMN-E, or MPO, making it less suitable for clinical use (Sugi *et al* 1996, Silberer *et al* 2005).

8.5 S100A12 protein

Fecal S100A12-protein, known earlier as calgranulin C, is more restricted to granulocytes than is calprotectin and is abundant at sites of inflammation in the intestinal mucosa of IBD patients (Foell *et al* 2003, 2008). It is secreted by activated neutrophils and promotes inflammation by binding to the receptor for advanced glycation endproducts (RAGE), leading to activation of NF-κB (Foell *et al* 2003). S100A12 is a non-specific marker of intestinal inflammation. Serum and mucosal levels of S100A12 are more elevated in IBD than in healthy controls (Leach *et al* 2007). According to a recent study, fecal S100A12 correlates better with intestinal inflammation than does fecal calprotectin or other biomarkers (Kaiser *et al* 2007). In distinguishing active IBD from IBS, for calprotectin a sensitivity of 63% and a specificity of 86% appeared. Correspondingly, for S100A12, sensitivity was 86%, and specificity was 96% (Kaiser *et al* 2007). In distinguishing pediatric IBD- from non-IBD patients, both calprotectin and S100A12 gave a high sensitivity (of 100% and 97%), but the specificity of S100A12 was better (97% versus 67%) (Sidler *et al* 2008).

8.6 Eosinophil granule-derived proteins

Fecal levels of eosinophil cationic protein and eosinophil protein X increase significantly in both active UC and clinically active CD (Saitoh *et al* 1999); in a 3-month follow-up, these proteins seem to predict relape in CD.

Aims of the study

The aims of the present study were to

1. study, in Crohn's disease, the correlation of fecal calprotectin and lactoferrin concentrations with
   - clinical and endoscopic disease activity (Study I)
   - histologic disease activity (Study II).

2. explore, during Crohn's disease anti-TNFα therapy,
   - changes in fecal calprotectin and lactoferrin concentrations (Study III)
   - parallel changes in mucosal effector and regulatory T-cell markers (Study IV).

Patients and methods

1 Patients

1.1 Cross-sectional Studies (I and II)

1.1.1 Patients in Study I

Between January 2005 and November 2006 in the Helsinki University Central Hospital clinic of gastroenterology, 84 adult CD patients referred for ileocolonoscopy were recruited. CD diagnosis was based on standard clinical, endoscopic, radiological, and histologic criteria. The Montreal classification served for CD classification (Silverberg *et al* 2005). Exclusion criteria were history of extensive bowel resection (ileosigmoideostomy, ileorectostomy), ostomy, long-term use of NSAIDs, pregnancy, or symptoms related mainly to perianal fistulating disease. Of the 84, five patients were excluded for failure to provide stool samples on time, one for positive fecal culture for *Campylobacter jejuni*, and one for canceling endoscopy.

The number of ileocolonoscopies was 106 for 77 patients (25 patients underwent endoscopy twice and two patients three times). Indications for endoscopy were assessment of endoscopic activity after medical treatment (n=35), clinically active disease (28), evaluation of endoscopic disease activity (20), stricture dilatation (9), postoperative evaluation (7), dysplasia surveillance for longstanding disease (7). Each patient provided a single stool sample (during the week before bowel preparation or the week after endoscopy) and underwent blood sampling for blood count, albumin, ESR, and CRP. Stool samples were stored at -40ºC until analysis. The 13 patients who provided their stool samples outside the scheduled week (8 to 13 days before bowel preparation) remained included because of unchanged disease course. The CDAI served for assessment of clinical activity (Best *et al* 1976): CDAI <150 indicated clinically inactive, and scores 150 to 219 mildly, 220 to 450 moderately, and >450 severely active disease (Sostegni *et al* 2003). Clinical characteristic of the patients are shown in Table 7.

Table 7. Clinical characteristics of the patients.

	n (%)
Patients	77
Gender, female/male	38/39 (49/51)
Age, years, median	33.5 (range 19-70)
Age at diagnosis, years	
<16	5 (6)
17-40	63 (82)
>40	9 (12)
Disease location	
ileum	19 (25)
colon	14 (18)
ileocolon	37 (48)
ileum + upper GI tract	3 (4)
colon + upper GI tract	0
ileocolon + upper GI tract	4 (5)
Disease behavior	
inflammatory	28 (36)
stricturing	29 (38)
penetrating	7 (9)
inflammatory + perianal disease	8 (10)
stricturing + perianal disease	4 (5)
penetrating + perianal disease	1 (1)
Mean duration of disease, years	9.2
Prior surgery: no/yes	44/33 (57/43)
Smoking: no/yes	51/26 (66/34)
Maintenance treatment/endoscopy	
no medication	12 (11)
aminosalicylate or sulfasalazin	73 (69)
thiopurine or methotrexate	72 (68)
metronidazole	8 (8)
corticosteroids	12 (11)
TNFα antagonist	14 (13)

GI, gastrointestinal. TNF, tumor necrosis factor.

1.1.2 Patients in Study II

We performed histology scoring for endoscopic findings of 67 patients who also participated in Study I. Two additional patients were included (a 52- and a 22-year-old woman). During the study period, 22 patients underwent ileocolonoscopy twice and two patients three times. Of 95 endoscopies performed, 8 remained incomplete due to a stricture or technical problems, and those were excluded. The overall number of

endoscopies analyzed in Study II was thus 87 performed on 61 patients. Findings of biopsy specimens were scored according to the histology score described below.

1.2 Prospective treatment Studies (III, IV)

1.2.1 Patients in Study III

Between January 2005 and March 2007, adults with Crohn´s disease and scheduled for ileocolonoscopy were recruited. Of these 15 patients, 13 patients were included also in cross-sectional Study I. Based on global clinical assessment, these patients were considered to need anti-TNFα therapy, indications being an acute flare (n=6), chronic active disease (6), or rapid reoccurrence of the disease postoperatively (3). Exclusion criteria were contra-indications for anti-TNFα treatment, pregnancy, ostomy, history of extensive bowel resection (ileosigmoideostomy, ileorectostomy), long-term use of NSAIDs, or perianal fistulating disease without luminal inflammation.

Of 15 patients receiving anti-TNFα therapy, 6 were females. Median disease duration was 5.1 (range 0.4-27) years. Active ileal disease occurred in two, colonic disease in four, and ileocolonic disease in nine patients; CD behavior was inflammatory±perianal disease in ten and stricturing in five patients.

Following the baseline endoscopy (median 7 days afterwards, range 1-38 days), anti-TNFα therapy was introduced: One patient received adalimumab induction (80 mg followed by 40 mg subcutaneously every other week until week 8) and 14 patients received infliximab induction (intravenous infusion 5 mg/kg at weeks 0 and 8). Corticosteroids were tapered off after introduction of anti-TNFα treatment, but the maintenance therapy remained unchanged. At the time of the first ileocolonoscopy, and at 2, 8, and 12 weeks (10 weeks for the adalimumab-treated patient) after start of therapy, patients provided fecal samples for measurements of calprotectin and lactoferrin and blood samples for serum CRP. At week 12 (week 10 for the adalimumab-treated patient), each patient underwent a post-treatment ileocolonoscopy for evaluation of endoscopic response and scoring of the CDEIS. Clinical activity was scored according to the CDAI (Best *et al* 1976).

1.2.2 Patients in Study IV

Of the 15 patients in Study III, 13 participated in Study IV. The control group for this study comprised 14 non-IBD patients who were referred for ileocolonoscopy for the following indications: change in bowel habits (n=3), diverticular disease (2), colorectal cancer follow-up (2), history of polyps (1) or adenoma (2), history of rectal bleeding (2), abdominal pain (1), and exclusion of CD in one patient with a history of perianal abscesses. In all control subjects, routine histologic analysis of ileal biopsies was normal.

2 Methods

2.1 Endoscopic scoring

Experienced gastroenterologists (T.S., H.N., U.T., M.F.) performed the ileocolonoscopies and graded findings according to the CDEIS (Mary *et al* 1989): The extent of surface showing disease, presence of mucosal superficial or deep ulcerations, and presence of either ulcerated or non-ulcerated stenosis were evaluated in each segment of the colon (right, transverse, left colon, and rectum) and ileum. The SES-CD (Daperno *et al* 2004) was also scored during all endoscopies and is decribed in more detail in the original publication (II).
No validated endoscopic limits to remission or to endoscopically mild, moderate, or severe activity exist. Thus, in Studies I and III, somewhat arbitrarily, CDEIS <3 suggested inactive, 3 to 9 mildly, ≥9 to 12 moderately active, and ≥12 severely active disease. In Studies III and IV, endoscopic treatment response was defined as a 2- or 3-class change in the CDEIS or achievement of remission (CDEIS <3). Partial response was defined as a 1-class improvement in endoscopic score.

2.2 Histology

2.2.1 Histology scoring

During all endoscopies, four biopsy specimens—targeted at the most severely diseased area—were taken from the ileum, right, transverse, and left colon, and the rectum. If no lesions were present in the segment, the biopsies were collected from random sites in each segment. Routine histology was performed on specimens stained with hematoxylin and eosin. In Studies II and III, a single experienced gastrointestinal pathologist (P.K.) scored the histologic findings according to the scoring system for histologic abnormalities in Crohn's disease mucosal biopsy specimens (D'Haens *et al* 1997, 1998). This scoring system is described in detail in original publications II and III.

2.2.2 Biopsy specimens in Study IV

During baseline and post-treatment endoscopies, two mucosal biopsy specimens were taken from the most severely diseased lesions from both the ileum and the colon for Study IV**.** If no lesions were present in the segment, the biopsies were collected from random sites in the ileum and colon. All samples were immediately deposited in cooled tubes including 0.9% saline solution and were delivered without delay to the laboratory and frozen at -70°C. Antibodies to CD4, CD8, IL-17, and Foxp3 served for immunohistochemical staining of the mucosal T cells. Positive cells comprising the lamina

propria were counted under a light microscope, followed by a quanitative real-time polymerase chain reaction (PCR) for detection of expression of IL-17A, Foxp3, IL-23A p19, and IFN-γ. Immunoenzymatic labeling, microscopic evaluation, and quantitative real-time PCR are described in more detail in the original publication IV.

2.3 Fecal calprotectin and lactoferrin assays and blood tests

Fecal calprotectin was measured by a quantitative enzyme immunoassay (PhiCal Test, Calpro AS, Oslo, Norway; NovaTec Immunodiagnostica, Dietzenbach, GmBH, Germany). Lactoferrin was measured by a quantitative enzyme immunoassay (IBD-SCAN, Inverness Medical, Princeton, NJ, USA; Techlab, Blacksburg, VA, USA). The upper limit of normal for fecal calprotectin was <100 µg/g, (Kolho *et al* 2006, von Roon *et al* 2007) and for fecal lactoferrin <7.25 µg/g of stool (manufacturer's baseline value, Kane *et al* 2003). Blood count, serum albumin, ESR, and CRP were determined as routine laboratory values.

2.4 Statistics

Each value is presented as a median. For data analyses we used the Statistical Package for the Social Sciences for Windows software 14.0 (SPSS, Chicago, IL, USA). Correlations were analyzed with two-tailed Spearman´s rank order correlation coefficient (r). For nonparametric tests, the Mann-Whitney and Kruskall-Wallis tests served in exploring associations between activity groups. The Wilcoxon signed-rank test was used for comparisons between paired samples. Significance was set at 0.05.

2.5 Ethical considerations

For participation in these studies, all patients gave their informed written consent, approved by the ethics committee of the Helsinki University Central Hospital.

Results

1 Cross-sectional studies

1. 1 Endoscopic activity and fecal markers

The ileum was intubated in 95 (90%) of endoscopies. Calprotectin samples numbered 106 and lactoferrin samples 104. In all samples, median fecal calprotectin was 178 µg/g (range 11 to 18575) and fecal lactoferrin 7.7 µg/g (range 0.0-2970.6). The CDEIS was 5.4 (0-26), the SES-CD was 7 (0-31, unpublished data for the SES-CD, Sipponen *et al*), and S-CRP (n= 105) was <5 mg/l (range <5-211). Correlations of the CDEIS with the SES-CD, fecal markers, and the CDAI are presented in Figure 1. The SES-CD correlated with calprotectin (r=0.699) and lactoferrin (r=0.751) significantly (p<0.001). Fecal calprotectin correlated significantly with fecal lactoferrin (r=0.864, p<0.001). Correlation of the CDEIS with CRP was significant (r=0.553, p<0.001). CRP correlated significantly with calprotectin (r=0.618, p<0.001) and lactoferrin (r=0.607, p<0.001) (Sipponen *et al*, unpublished data).

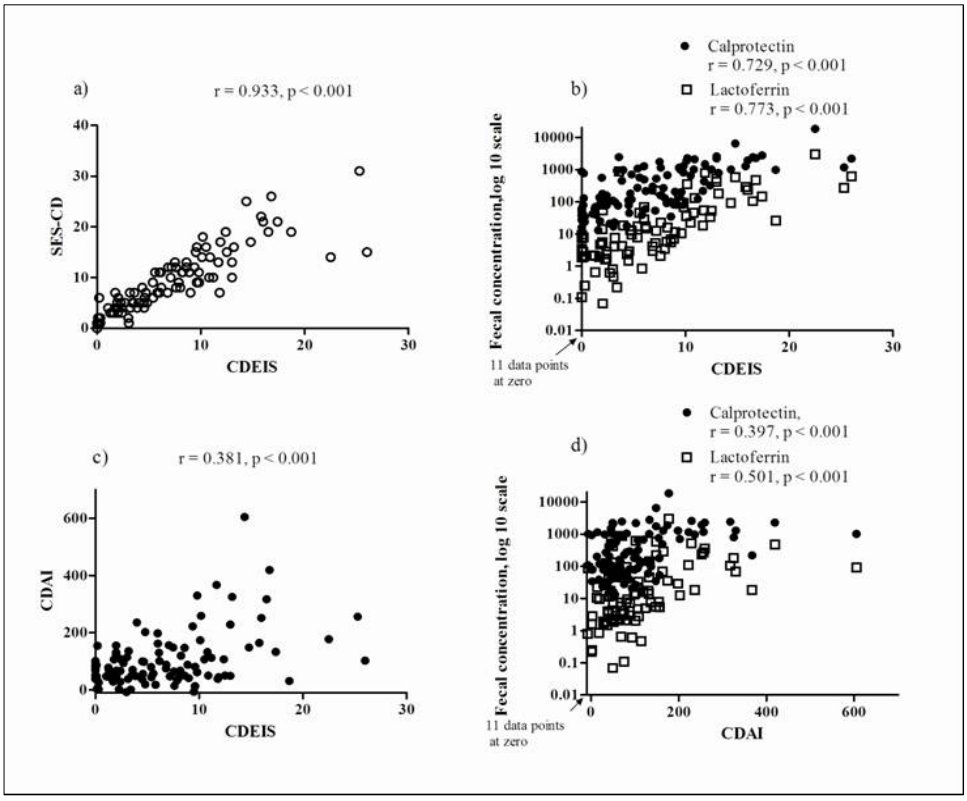

Figure 1. *Correlations of a) the CDEIS with the SES-CD, b) the CDEIS with fecal calprotectin and lactoferrin, c) the CDEIS with the CDAI, and d) the CDAI with fecal*

calprotectin and lactoferrin. CDEIS, Crohn's disease index of severity; SES-CD simple endoscopic score for Crohn's disease; CDAI, Crohn's disease activity index

1.1.1 Fecal markers according to endoscopic disease activity

Endoscopic activity was classified according to CDEIS score into inactive (CDEIS 0 to 3, n=36, 34%), mildly active (CDEIS ≥3 to 9, n=38, 36%), and moderately (CDEIS ≥9 to 12, n=16, 15%) or severely active disease (CDEIS ≥12, n=16, 15%) disease. Fecal calprotectin and lactoferrin levels were significantly lower in patients with inactive than with active disease (CDEIS ≥3) ($p<0.001$).

Endoscopically inactive disease
In inactive disease, median calprotectin concentration was 63 µg/g (range 11-869), lactoferrin 1.9 µg/g (0.0-81.3), CDAI 60 (2-155), SES-CD 3 (0-7). Both fecal markers were significantly lower in this group than in mildly, moderately, or severely active disease (all $p<0.001$) Both fecal markers were normal in 24 of 36 (67%), calprotectin was normal in 25 (69%) and lactoferrin in 31 (86%) patients; 16 (44%) of calprotectin values were even below the manufacturer's reference limit for normal (50 µg/g). Despite endoscopically inactive disease, both fecal markers were elevated in five (14%) patients: upper-GI disease occurred in three patients, one had erosive pseudopolyps in the colon, and one had a superficial ileocolonic anastomosis ulcer.

Endoscopy revealed no ulcers in 22 patients (66%) in the endoscopically inactive group. Of these, the majority, 18 (82%) had either a normal calprotectin or lactoferrin concentration, and both fecal markers were normal in 14 (64%) patients.

Endoscopically mild disease
Of 106 endoscopies, 38 (36%) indicated endoscopically mildly active disease. Median calprotectin concentration was 170 µg/g (range 17 to 2440), lactoferrin 6.8 µg/g (0.0 to 891.3), CDAI 69 (-8 to 236), and SES-CD 8 (1 to 13). In mildy active disease, fecal calprotectin and lactoferrin values differed significantly from those in endoscopically moderate or severe disease (all $p<0.001$). In 25 samples (66%), calprotectin concentration was abnormal. Lactoferrin was available for 37 patients and was elevated in 17 (46%) samples. Despite mild endoscopic activity, both fecal markers were normal in 11 (29%) patients (8 of these had either an ulcerated or a fibrotic stricture in the ileocolonic anastomosis). On the other hand, although endoscopic activity was considered mild, five patients (13%) had a considerably elevated calprotectin (>1000 µg/g) and two (5%) a high lactoferrin (>100 µg/g) concentration.

Endoscopicaly moderate or severe disease
In patients with endoscopically moderately or severely active disease (n=32), all fecal calprotectin and lactoferrin values were abnormal. Median calprotectin value was 1158 µg/g (123 to 18575), lactoferrin 105 µg/g (10.6 to 2970.6), CDAI 133 (-7 to 605), and SES-CD 15 (7 to 31). Of the 32, 22 (69%) had a calprotectin concentration over 1000 µg/g

and 16 (50%) a lactoferrin concentration over 100 µg/g. Based on the CDAI, 16 (56%) had clinically inactive disease, although their endoscopic activity was considered moderate or severe. Median SES-CD in this group was 15 (range 7 to 31). Compared to levels in inactive or mildly active disease, fecal calprotectin and lactoferrin were significantly higher in moderately or severely active disease ($p<0.001$).

1.1.2 Fecal markers according to disease location

Of the whole study group, those with strictly ileal disease (n=22) showed a significant correlation of their CDEIS with lactoferrin ($r=0.678$, $p<0.001$), but not with calprotectin ($r=0.316$, $p=0.151$). Interestingly, when purely ileal and ileal + upper GI tract disease were combined (n=28), calprotectin also correlated weakly with the CDEIS ($r=0.434$, $p=0.021$), but not with the SES-CD ($r=0.317$, $p=0.101$) (for SES-CD unpublished data, Sipponen T *et al*). Correlations of lactoferrin with the CDEIS ($r=0.525$, $p=0.005$) and SES-CD were significant ($r=0.485$, $p=0.01$). In the subgroup of patients with an intubated ileum and histologic analysis available (n=21), however, fecal markers in ileal ± upper GI tract disease failed to correlate with ileal SES-CD (for calprotectin $r=0.317$ and for lactoferrin $r=0.180$, $p>0.05$).

In colonic or ileocolonic CD ± upper GI tract disease (n=78), both calprotectin and lactoferrin correlated significantly with endoscopic scores (calprotectin with the CDEIS $r=0.770$, with the SES-CD $r=0.758$, lactoferrin with the CDEIS $r=0.805$, with the SES-CD $r=0.781$, all $p<0.001$). Table 8 presents median fecal-marker concentrations by disease location and activity.

In all location groups with upper GI-tract data pooled, fecal calprotectin was significantly higher in active than in inactive disease. Lactoferrin was significantly higher in active ileocolonic or colonic disease than in inactive, but not in ileal disease. Fecal markers were significantly higher in active colonic (both $p<0.001$) or ileocolonic (calprotectin $p=0.028$, lactoferrin $p=0.004$) than in ileal disease. In inactive disease, concentration differences in fecal markers between disease locations were non-significant.

Table 8. Fecal markers by disease location and activity.

Disease (number)	*Fecal calprotectin µg/g (range)*	*Fecal lactoferrin µg/g (range)*
Ileal disease*		
active disease (19)	180 (35-1294)	7.7 (0.0-68.5) (n=18)
inactive disease (9)	62 (37-156), p=0.025	2.1 (0.0-54.1), p=0.117
Colonic disease		
active disease (14)	1383 (45-6543)	179.4 (2.9-802.1) n=13
inactive disease (8)	41 (21-136), p<0.001	0.04 (0.0-1.9), p<0.001
Ileocolonic disease**		
active disease (37)	609 (17-18575)	29.9 (0.0-2970.6)
inactive disease (19)	63 (11-869), p<0.001	2.1 (0.0-81.3), p<0.001

Active disease: CDEIS ≥3. Median values.
* active group, 4 patients, inactive group, 2 patients had known upper GI-tract disease. ** active group, 3 patients, inactive group, 3 patients had known upper GI-tract disease.

1.1.3 Sensitivity, specificity, PPV, and NPV of fecal markers in predicting endoscopically active disease

Among the sensitivity, specificity, PPV, and NPV values of fecal markers, the CDAI, and CRP, values with a cutoff 80 for the CDAI and 10 mg/l for CRP are previously unpublished (Table 9). In predicting endoscopically moderately or severely active disease (CDEIS ≥ 9), sensitivity, specificity, PPV, and NPV for fecal calprotectin ≥1000 µg/g and for lactoferrin ≥50 µg/g were 69%, 93%, 82%, and 87%, and 65%, 96%, 87%, and 83%, respectively.

Table 9. Sensitivity, specificity, positive predictive value PPV, and negative predictive value NPV of variables in detecting endoscopically active disease (CDEIS ≥ 3).

	Sensitivity %	*Specificity* %	*PPV* %	*NPV* %
F-calprotectin ≥100 µg/g	81	69	84	66
F-calprotectin ≥200 µg/g	70	92	94	61
F-lactoferrin ≥7.25 µg/g	71	83	89	60
F-lactoferrin ≥10 µg/g	66	92	94	59
CDAI ≥80	56	65	78	46
CDAI ≥150	27	94	91	40
S-CRP ≥5 mg/l	48	91	91	48
S-CRP ≥10 mg/l	29	95	91	44

1.2 Clinical activity and fecal markers

The median CDAI in the study group was 74 (range -8 to 613). Correlations of the CDAI with the CDEIS and fecal markers are presented in Figure 1. The CDAI correlated significantly also with the SES-CD (r=0.373, p<0.001), but correlations of fecal markers with endoscopic scores were stronger. In Table 10, endoscopic scores and fecal markers are grouped according to clinical activity. According to the CDAI, 85 patients had inactive disease (CDAI <150), and 21 had active disease (CDAI ≥150). Although the CD was clinically inactive, fecal calprotectin was elevated (>100 µg/g) in 48 of 85 (56%) and lactoferrin in 33 of 83 (40%) patients. In clinically inactive disease, fecal calprotectin was considerably elevated (>1000 µg/g) in 14 (16%) patients and lactoferrin (>100 µg/g) in 8 (10%). The majority of the 21 patients with clinically active disease, however, had abnormal calprotectin (n=20; 95%) and lactoferrin (n=19; 90%) concentrations.

Table 10. Endoscopic scores and fecal markers by clinical disease activity.

CDAI	*CDEIS*	*SES-CD*	*Fecal calprotectin µg/g*	*Fecal lactoferrin µg/g*
<150	4.4 (0.0-26.0)	6 (0-21)	127 (11-6453)	4.2 (0.0-891.3)
≥150	10.2 (0.2-25.3)*	13 (4-31)*	1173 (53-18575)*	92.4 (5.3-2970.6)*

Values presented as median (range). * p<0.001 between clinical activity groups.

1.4 Histologic findings and fecal markers

The histologic score was available for 87 endoscopies. Calprotectin results numbered 87 and lactoferrin 85. In the whole study group, median total histology score was 12 (range 0-45), colon score 7 (0-38), and ileum score 4 (0-11). For ileal disease alone, ileal histology score was 6 (0-11). In ileocolonic or colonic disease, colon histology score was 13 (0-38) and ileal score was 4 (0-11). Of 87 endoscopies, 15 SES-CD values were ≤3 and only two of these had signs of increased polymorphonuclear cells in the epithelium or lamina propria (Sipponen *et al*, unpublished data).

Although ileal histology score correlated significantly with ileal endoscopic score, it failed to correlate with fecal markers. In ileocolonic or colonic disease, correlations of fecal markers with histology score were significant (Table 11).

Table 11. Correlations of fecal markers with the SES-CD and histologic scores.

	Fecal calprotectin		*Fecal lactoferrin*	
	ileal disease	*ileocolonic/colonic*	*ileal*	*ileocolonic/colonic*
Total SES-CD	0.050	0.733**	0.280	0.748**
ileal SES-CD	0.317	0.171	0.180	0.226
colonic SES-CD	-	0.642**	-	0.627**
SES-CD variables :				
presence of ulcers	0.016	0.670**	0.483*	0.666**
extent of ulcerated surface	0.010	0.688**	0.289	0.697**
extent of affected surface	0.251	0.645**	0.113	0.684**
presence of narrowings	-0.094	0.141	0.045	0.178
Total histology score	0.147	0.617**	-0.008	0.611**
ileal histology	0.311	0.178	0.291	0.255*
colon histology	-	0.563**	-	0.543**

* $p<0.05$; ** $p<0.001$.

1.4.1 Histology in patients with normal fecal-marker concentration

Patients with normal fecal calprotectin or lactoferrin had significantly lower endoscopic and histologic scores than did those with elevated fecal markers: total SES-CD ($p<0.001$), colon SES-CD ($p=0.004$ for calprotectin, $p<0.001$ for lactoferrin), total histology score ($p<0.001$), and colon histology score ($p=0.002$).

Ileal disease
Of 21 patients with ileal CD, 10 had normal fecal calprotectin, and 13 had normal lactoferrin. Compared to patients with elevated fecal markers, no significant differences occurred in ileal SES-CD or ileal histology scores (all $p>0.05$). The four patients with normal lactoferrin and elevated calprotectin showed an increased number of both polymorphonuclear neutrophils and mononuclear cells in the lamina propria and of polymorphonuclear cells in the epithelium. Of the patients with normal calprotectin, half had no or only architectural changes in the ileal biopsy specimens.

Colonic or ileocolonic disease
Of 66 patients with ileocolonic or colonic disease, calprotectin was normal in 21 and lactoferrin in 26. With normal calprotectin, median colon SES-CD was 3 (range 0-7), and colon histology score was 5 (0-25), both significantly ($p<0.001$) lower than in the 45 patients with an elevated calprotectin (colon SES-CD 9, range 0-26; colon histology score 18, range 0-38). Accordingly, in patients with normal lactoferrin, colon SES-CD was 3 (0-9) and histology score 5 (0-25), and both of these were significantly ($p<0.001$) lower than in those with elevated lactoferrin values (n=39, colon SES-CD 10, range 0-26, and colon histology score 18, range 0-38).

Nine biopsy specimens showed either no changes or architectural changes only. Of these, calprotectin was mildly elevated (140 µg/g) in one, whereas lactoferrin was normal in all nine. Furthermore, of 21 patients with normal calprotectin, 11 (52%) showed no histologic changes or showed architectural changes only.

2 Treatment studies

2.1 Fecal markers and endoscopic findings during anti-TNFα therapy

Of 30 endoscopies, the ileum was reachable in 28. Before the introduction of anti-TNFα therapy, the CDEIS indicated mildly or moderately active (CDEIS 3-12) disease in five patients (33%) and severe (CDEIS ≥12) in nine (60%). One patient with active disease in the neoterminal ileum had a low baseline CDEIS (1.8). As a result of therapy, both fecal markers, S-CRP, endoscopic, and clinical scores declined significantly from their baseline levels (Table 12).

Of 15 patients, 11 (73%) were endoscopically defined as treatment responders, one was a partial responder, and 3 were nonresponders. Of responders, five achieved remission (CDEIS <3). Their fecal calprotectin declined from 1891 µg/g (range 813-2434) to 27 µg/g (13-130) and lactoferrin from 92.4 µg/g (35.5-235.6) to 1.9 µg/g (0.0-2.1).

Changes in CDEIS (ΔCDEIS) correlated with ΔCDAI (r=0.679, p=0.005), Δcalprotectin (r=0.561, p=0.030), and Δlactoferrin (r=0.600, p=0.023), but failed to correlate with Δileal histology score (r=0.058, p=0.845), or Δcolon histology score (r=0.369, p=0.090). Correlations of Δcalprotectin and Δlactoferrin concentrations with Δileal histology score or Δcolon histology score were also nonsignificant (p>0.05).

2.2 Changes in mucosal T cell markers during anti-TNFα therapy

Compared to controls both before and after therapy, the number of IL-17$^+$ cells in ileal (before therapy p<0.0005 and after p=0.0005) or colonic specimens (p=0.015 and p=0.042, respectively) was increased. Moreover, the number of Foxp3$^+$ cells was increased compared to control numbers in ileal specimens before (p=0.023) and after (p=0.005) therapy. In colonic specimens, the number of Foxp3$^+$ cells was increased compared to controls only after therapy (p=0.032). During anti-TNFα therapy, changes in the number of IL-17$^+$ or Foxp3$^+$ cells in ileal or colonic specimens were nonsignificant (all p>0.05). In ileal or colonic specimens, the number of CD4$^+$ or CD8$^+$ cells showed no significant difference before or after the therapy between the groups (all p>0.05). Table 13 shows the changes in mucosal IL-17, IL-23, Foxp3, and IFN-γ specific messenger ribonucleic acid (mRNA) expression in ileal and colonic biopsy specimens compared to controls.

In ileal specimens, the relation of IL-17$^+$ cells to CD4$^+$ cells (calculated for each individual) was higher than among control subjects both at 0 and 3 months (p=0.006 and p=0.0013). A significant decrease occurred in the relation of IL-17$^+$ cells to CD4$^+$ cells after introduction of anti-TNFα treatment (p=0.047). The relation of IL-17$^+$ cells to Foxp3$^+$ cells was higher in the patients' baseline samples than in the post-treatment specimens (p=0.038).

At baseline, the ileal biopsy specimens showed no significant correlations between FoxP3$^+$ or CD4$^+$ cells and the CDEIS or between number of IL-17$^+$ cells or IL-17 mRNA expression and the CDEIS. After anti-TNFα induction, however, the number of IL-17$^+$ cells and the CDEIS (r=0.806, p=0.007) and IL-17 mRNA expression and the CDEIS (r=0.736, p=0.038) showed a close correlation. The number of CD8$^+$ cells showed a positive correlation with the CDEIS after the therapy, but not in the baseline samples. No significant correlation of calprotectin with IL-17$^+$ cells was detectable (r=0.683, p>0.05).

Table 12. Fecal markers, endoscopic, histologic, and clinical scores and S-CRP during anti-TNFα therapy. Values before therapy compared to values at 12 weeks.

	Before therapy	*2 weeks*	*8 weeks*	*12 weeks*
CDEIS	13.0 (1.8-25.3)	-	-	4.8 (0.0-11.2) p=0.002
Colon histology	23 (0-38)	-	-	11 (0-25) p=0.002
Ileum histology	7 (0-11)	-	-	4 (0-9) p=0.022
CDAI	158 (49-605)	77 (13-197)	68 (26-192)	66 (24-202) p=0.005
F-calprotectin µg/g	1173 (88-15326)	216 (24-2349)	325 (15-20899) n= 14	130 (13-1419) p=0.001
F-lactoferrin µg/g	105.0 (4.2-1258.9)	6.7 (0.9-356.8)	7.1 (0.0-5400.6) n= 12	2.7 (0.0-228.5) n= 14 p=0.001
S-CRP mg/l	10 (< 5-54) n=14	< 5 (< 5-11)	< 5 (< 5-19)	< 5 (< 5-6) p=0.005

Median values (range). CDEIS, Crohn's disease index of severity; CDAI, Crohn's disease activity index.

Table 13. IL-17-, IL-23-, Foxp3-, and IFN-γ-specific mRNA expression in ileal and colonic biopsy samples taken before and 3 months after start of anti-TNFα-therapy. Values compared to controls. Median (range) mRNA levels expressed as relative units for each group.

	before therapy	*at 3 months*	*Controls*
Ileum			
IL-17	128.0 (30-325)	67.5 (30-615)	30.0 (30-419)
IL-23	373.0 (322-804)*	444.5 (235-542)*	225.0 (153-706)
Foxp3	611.0 (15-792)*	104.5 (15-891)*	20.5 (15-220)
IFN-γ	19.6 (8.6-230)***	14.0 (1.0-37.6)*	1.0 (1.0-6.0)
Colon			
IL-17	133.5 (30-588)*	79.0 (30-1607)	30.0 (30-144)
IL-23	167.0 (72-405)	240.0 (74-983)	252.0 (9-392)
Foxp3	311.5 (63-694)**	135.5 (15-1284)	49.0 (15-252)
IFN-γ	45.0 (2.0-249.7)**	15.7 (1.0-122.2)*	1.0 (1.0-5.6)

*$p<0.05$, **$p<0.01$, ***$p<0.001$.

Discussion

1 Fecal markers as surrogate markers of endoscopic activity

Our study showed that fecal calprotectin and lactoferrin correlated closely with the validated endoscopic scores CDEIS and SES-CD. These correlations were stronger than were correlations of endoscopic scores with clinical acitivity assessed with the CDAI or with CRP. Furthermore, in endoscopically inactive disease (CDEIS <3) both fecal markers were significantly lower than in endoscopically mild, moderate, or severe disease.

Several studies have shown fecal calprotectin to be higher in clinically active CD than in inactive disease (Tibble *et al* 2000a, 2002, Costa *et al* 2003, Carroccio *et al* 2003, Dolwani *et al* 2004, Wassell *et al* 2004, Gaya *et al* 2005, Scarpa *et al* 2007, Schröder *et al* 2007). Similarly, fecal lactoferrin has been elevated in clinically active disease (Uchida *et al* 1994, Kayazawa *et al* 2002, Kane *et al* 2003, Dai *et al* 2007, Schröder *et al* 2007, Walker *et al* 2007). In these studies and in studies detecting fecal calprotectin concentrations in clinical CD remission (Roseth *et al* 1999, Tibble *et al* 2000b, Costa *et al* 2005), fecal-marker concentrations have been elevated even in clinically inactive disease. This is in keeping with earlier studies that have detected, based on fecal excretion of ^{111}In-labeled granulocytes, low-grade inflammation in symptomless CD patients (Saverymuttu 1986) and poor correlation of their clinical activity score CDAI with endoscopic findings (Modigliani *et al* 1990, Cellier *et al* 1994). In our study, almost all patients with clinically active disease had abnormal fecal markers, confirming that their symptoms were most probably a result of intestinal inflammation and not a sign of noninflammatory conditions (such as functional symptoms, stenosis, or bile acid malabsorption). Fecal markers were, however, abnormal in a considerable number of patients with clinically inactive disease and were in some of these patients even highly elevated. The CDAI seems thus to underestimate mucosal inflammation, and a remission based only on the CDAI cannot be intepreted as complete. An adjusted cutoff of the CDAI (>80) has been shown to improve diagnostic accuracy (Langhorst *et al* 2008). In the present study, a similar adjusted cutoff doubled the sensitivity of CDAI in detecting endoscopically active disease, but this remained still lower than the sensitivities of fecal markers.

Crohn's disease is not associated with perforating complications or fibrostenosis early in its onset. The majority of CD patients start with inflammatory lesions. Continuing inflammation in symptomless patients may indicate a stage of active ongoing inflammation which can progress to clinical relapse or complications. On the other hand, achievement of mucosal healing appears to correlate with fewer hospitalizations (Rutgeerts *et al* 2006). Therefore, detection of intestinal inflammation even in nonsymptomatic patients is valuable. Finding reliable surrogate markers for endoscopic activity, especially in CD, is thus important. One of the first studies showing the importance of fecal calprotectin as a surrogate marker of endoscopic activity was Roseth and coworkers' report (1997) showing in UC patients a close correlation of fecal calprotectin with endoscopic and histologic activity. Studies showing calprotectin to be significantly elevated in IBD or CD patients compared to those with nonorganic disease have performed endoscopies, but CD findings

were not scored with a validated endoscopic index (Limburg *et al* 2000, Tibble e*t al* 2000a, 2002, Schröder *et al* 2007). In a study comparing calprotectin and S100A12 in detecting inflammation, endoscopic findings of CD patients were scored according to the SES-CD, but the histology score served as the reference standard for absence or presence of inflammation, and correlations of fecal markers with the SES-CD results were not published (Kaiser *et al* 2007). A recent study of 164 CD patients scored endoscopic findings according to the SES-CD and showed a significant correlation of both fecal markes with endoscopic disease activity (Jones *et al* 2008). The SES-CD served as an endoscopic score also in two studies of Schoepfer and coworkers (2007, 2008). In moderately or severely endoscopically active CD (SES-CD ≥20), calprotectin as well as lactoferrin was significantly higher than in mild or inactive disease (Schoepfer *et al* 2008). Two studies grading endoscopic findings of both UC and CD on a scale from 0 to 3 (from "no inflammation" to mild, moderate, or severe inflammation) showed a close correlation of endoscopic activity with fecal calprotectin (Langhorst *et al* 2008, D'Inca *et al* 2007). In Langhorst and coworkers' research (2008), lactoferrin also correlated closely with endoscopic inflammation in CD. In D'Inca and coworkers' research (2007), lactoferrin—analyzed with a qualitative ELISA—correlated with histologic score, but not with endoscopic activity.

In the present study detecting endoscopic findings of a higher number of CD patients, both fecal markers showed a close correlation with endoscopic disease activity as defined by the CDEIS or SES-CD. A different finding was published by Denis and coworkers (2007): With a CDEIS cutoff of ≤6 for endoscopically mild disease, their 28 patients with clinically active CD and a normal CRP failed to show any correlation of their CDEIS scores with fecal calprotectin. In that study over half the patients had pure ileal disease in which the CDEIS upper limit of 6 is probably too high for minor activity. In our study, with a larger population showing a positive correlation of fecal markers with endoscopic grading, the cutoff for endoscopically inactive disease was set lower (CDEIS <3), and mild activity was scored between 3 and 9.

Although the CDEIS has served in several treatment studies, endoscopic remission in CD is not clearly defined, and no specific numeric cutoff CDEIS or SES-CD value for remission has been determined (Sandborn *et al* 2002, Hommes *et al* 2004, Rutgeerts *et al* 2007). In patients achieving clinical remission after corticosteroid treatment, the CDEIS was 6.9±5.5 (mean ± standard error of mean), and in those achieving both endoscopic (no lesions or only scars, or minor lesions with at least "a 2-grade decrease on the 5-grade scale") and clinical remission it was 2.1±1.7 (Modigliani *et al* 1990). In another work, patients in endoscopic remission (no lesions or scarred lesions only or "minor lesions with at least a two-grade decrease on the six-grade scale of endoscopic severity and no residual deep ulcers") after 3 to 7 weeks' steroid therapy had a CDEIS of 2.0±0.3, and patients in clinical but not endoscopic remission achieved a CDEIS 5.1±0.7 after prolonged steroid therapy (Landi *et al* 1992). A study by Mary and coworkers (2005)—published as an abstract—revisited the data collected for validation of the CDEIS; it suggested a CDEIS cutoff value for endoscopic remission of between six and seven (D´Haens 2007). CDEIS values close that level were achieved in patients treated with a single 5 mg/kg infliximab infusion: the CDEIS declined from baseline level 15.1±6.9 to 6.4±5.1 at week four

(D'Haens *et al* 1999). Accordingly, patients treated with a three-dose induction with infliximab showed a significant reduction in the CDEIS at week 10, and with maintenance therapy (every eighth week) at week 54 (median CDEIS was under 3) (Geboes *et al* 2005). In that trial, a CDEIS less than 5 was considered mild disease, 5 to 15 moderate, and >15 severe disease.

Some infliximab-response studies define complete mucosal healing as "complete absence of mucosal ulcers observed at baseline" (Rutgeerts *et al* 2004, 2006). When endoscopic remission is defined thus, even having one single aphthous ulcer in otherwise healed mucosa is not considered remission. Ideally, only a CDEIS as low as zero should be considered complete endoscopic remission. In the present study, for practical reasons, we decided to give a numeric cutoff for remission (CDEIS <3). This allows minor changes in CD mucosa in endoscopic remission. It is comparable to CDEIS values in endoscopic remission from Modigliani and Landi *et al*'s studies described above (Modigliani *et al* 1990, Landi *et al* 1992), but considerably lower than the cutoff suggested by Mary and coworkers (2005). Accordingly, a SES-CD <3 was defined as endoscopically inactive disease. With these cutoff values for CDEIS and SES-CD, we showed fecal neurophil-derived proteins to be significantly lower in endoscopic remission than in active disease. A CDEIS <3 for remission served also in our anti-TNFα study.

A normal fecal-marker value is possible to achieve even in CD. In the present study, the majority of CD patients with inactive disease had a normal fecal calprotectin or lactoferrin concentration. In detecting endoscopically active disease, the sensitivity of a normal fecal-marker level (50 or 100 μg/g) is good, but the specificity is lower. Clinical practice may require an adjusted upper limit for normal fecal-marker concentration in CD. We found reasonable sensitivity and a good specificity and PPV for active disease with a cutoff value for calprotectin at 200 μg/g and for lactoferrin at 10 μg/g, but the negative predictive value of these cutoffs was less satisfactory. In detecting moderate or severe endoscopic activity, cutoff values for calprotectin of 1000 μg/g and lactoferrin of 50 μg/g had fairly good NPV. In clinical practice, a low fecal-marker value excludes active inflammation, and with a considerably elevated value, active inflammation is very likely. These markers are, however, not ideal, and in future other proteins such as S100A12 may even replace calprotectin and lactoferrin, but more studies are needed for confirmation (Kaiser *et al* 2007).

Compared to endoscopy, one benefit of neutrophil-derived proteins is the ability to detect inflammation throughout the GI tract. When ileocolonoscopy fails to show any inflammation, and a fecal-marker concentration is elevated, the upper GI tract and small bowel reguire examination. We did not examine the upper GI tract systematically during our study, but any known upper-GI tract disease was classified according to the Montreal classification (Silverberg *et al* 2005). For ileal ± upper-GI disease our results are somewhat controversial; it seems, however, that in ileal CD with limited disease extension, fecal calprotectin and lactoferrin are not as clearly elevated as in colonic or ileocolonic disease. A study from Mayo Clinic supports our findings: Correlations between fecal markers and isolated ileal disease were at best weakly positive, but were strong in colonic disease (Jones *et al* 2008). A similar but nonsignificant trend at least with lactoferrin and PMN-E

emerged in Schröder and coworkers' study (2007). Calprotectin also correlates with disease extent and severity (Bunn *et al* 2001b, Fagerberg *et al* 2007).

A different result emerged in research on postoperative CD, where fecal-marker concentrations in ileal involvement were similar to concentrations in anastomotic or colonic involvement (Scarpa *et al* 2007). Moreover, a study including 49 CD patients (27 with ileal CD, 6 with colonic, and 16 with ileocolonic disease) failed to reveal any influence of disease site on calprotectin values (Costa *et al* 2003), and another study, one of pediatric patients, showed a non-significant fecal calprotectin difference between ileal CD and colonic or ileocolonic disease (Bremner *et al* 2005). Furthermore, serum or mucosal (achieved from the cecum only) calprotectin levels between disease-location groups were similar (Leach *et al* 2007). A recent study, however, showed that release of calprotectin and S100A12 protein was strongly dependent on disease location in endoscopically and histologically active CD: In ileal disease, release was minor from sites of inflammation compared to release in colonic inflammation (Foell *et al* 2008). This may explain our differing results by disease location. The small number of ileal CD patients, with the majority of them having a stricturing phenotype—although endoscopic and histologic inflammation was detectable—may bias the results. A larger study is needed of fecal markers in small bowel CD or strictly ileal CD.

In the present study, fecal calprotectin and lactoferrin gave fairly similar results in detecting endoscopically active disease. A study finding lactoferrin correlating better with CRP than does calprotectin suggests that lactoferrin reflects more systemic inflammation but calprotectin reflects a local phenomenon (Scarpa *et al* 2007). This was speculated to be a consequence of differences in the release mechanisms of these two proteins. Although lactoferrin is a neutrophil-degranulation protein, and calprotectin is among the DAMPs, the present study does not support Scarpa *et al*'s finding (2007), as both fecal markers correlated significantly with CRP. Combination of calprotectin with other neutrophil-derived markers (lactoferrin or PMN-E) seems not to improve the diagnostic accuracy of calprotectin (Schröder *et al* 2007).

Our study included only patients with known CD. It does not answer the question whether fecal markers should be included in the diagnostic workup of patients complaining of abdominal symptoms (diarrhea, lower abdominal pain). Data from several studies, however, support this strategy (Tibble *et al* 2000a, Carroccio *et al* 2003, Costa *et al* 2003, D'Inca *et al* 2007, Schoepfer *et al* 2007, 2008). In a clinical setting, it seems that detection of fecal calprotectin or lactoferrin would be of more benefit than measurement of ESR or CRP—tests often included in the diagnostic workup of patients with abdominal symptoms. It is suggested that the diagnosis of IBD can be excluded in patients who have a negative calprotectin test and who fulfill the Rome criteria of IBS (Aadland *et al* 2002). Those with an elevated concentration, once infection is ruled out, could be referred to endoscopy. This strategy could reduce the number of endoscopy referrals, and on the other hand, possibly shorten the interval between IBD symptoms and diagnosis. What should be noticed, however, is that patients with alarming symptoms or findings like anemia, weight loss, or rectal bleeding are likely to need endoscopic investigation regardless of fecal-marker values.

It should be remembered that fecal markers cannot replace IBD patients' surveillance endoscopies performed for detection of colonic dysplasia. Possibly fecal markers could, however, aid in better timing of these endoscopies: the ideal time for surveillance colonoscopy would be when fecal marker concentration is low, making possible dysplastic findings easier to detect.

2 Fecal markers and histologic activity

The present study showed that fecal calprotectin and lactoferrin concentrations correlated significantly with histologic activity in ileocolonic and colonic disease. The majority of patients with endoscopically inactive disease had no signs of increased infiltration of neutrophils in the epithelium or lamina propria. Furthermore, fecal markers were higher in the more severe histologic findings than in those with lower histologic activity. No such difference was evident in ileal disease.

In pediatric and adult IBD, fecal calprotectin correlates with histologic grades of inflammation (Roseth *et al* 1997, Limburg *et al* 2000, Bunn *et al* 2001b), and in histologic remission, fecal calprotectin has been significantly lower than in histologically active CD (Fagerberg *et al* 2007, Canani *et al* 2008). Furthermore, low calprotectin levels have been detectable in patients with histologic remission (Roseth *et al* 1997, Fagerberg *et al* 2007). In keeping with this, in the present study, half the patients with a normal calprotectin value had no signs of inflammation in their biopsy specimens. In a pediatric IBD study, a calprotectin cutoff value of 85.7 μg/g gave a sensitivity of 93% and a specificity of 82% in detecting microsopically active colonic inflammation (Fagerberg *et al* 2007). In another study, a higher calprotectin cutoff value of 143 μg/g has been suggested for discrimination in IBD between histologically active disease and remission (Canani *et al* 2008). When histologic relapse was studied retrospectively in 73 quiescent IBD patients, for the 32 presenting with histologic relapse, a calprotectin value of 275 μg/g was a sensitive and specific marker (Diamanti *et al* 2008). In that study, of 18 nonrelapsed CD patients, 5 presented with a fecal calprotectin concentration over 275 μg/g and 4 of these had small bowel disease. Changes according to disease location were, however, statistifically nonsignificant (Diamanti *et al* 2008).

Histologic remission is not clearly defined (Sandborn *et al* 2002). Biopsy specimens represent only a tiny area of the lesion from which they come, and a sampling error may bias the results in either direction—toward greater or lesser severity. Sampling error may be the reason for the present anti-TNFα study's failing to show a significant correlation of changes in histologic score with changes in endoscopic score or with fecal calprotectin and lactoferrin concentrations. Fecal markers seem to represent the global intestinal inflammation better than do the biopsy specimens. Obtaining biopsy specimens only for estimation of CD activity thus may be unnecessary.

3 Fecal markers in monitoring therapy

Calprotectin has been shown to predict mucosal healing in IBD (Roseth *et al* 2004). In that study, IBD patients in clinical remission with a normal calprotectin concentration (<50 mg/l) were included. In all except one patient, the endoscopic appearance of the mucosa was normal, and in the majority histologic findings also showed complete mucosal healing. Median calprotectin value in CD patients was 35 mg/l. A small number of CD patients (n= 6) had calprotectin data from a previous active phase, and these values were significantly higher than in remission. A decline in fecal calprotectin during infliximab therapy has been reported in two patients (Aadland and Fagerhol 2002) and in fecal lactoferrin in five pediatric patients (Buderus *et al* 2004). Our prospective study supports these results: It showed a significant decline in both fecal markers during anti-TNFα therapy and normalization of markers in patients achieving endoscopic remission.

Objective monitoring of treatment success is important, especially when treatment requires expensive biological drugs. Based on our findings, fecal calprotectin and lactoferrin are reliable surrogate markers of mucosal healing. During anti-TNFα therapy, endoscopic monitoring of CD activity can be replaced with measurements of either of the fecal neutrophil-derived markers. The one exception may be a patient with ileal disease and normal baseline fecal-marker concentration.

During clinical trials, normalization of the CDAI still widely serves as a primary endpoint of succesful therapy. Rather than the CDAI, fecal neutrophil-derived proteins, however, might serve as objective markers of disease activity. In Study III, calprotectin more than 200 μg/g and lactoferrin >10 μg/g served as surrogate markers of active disease. Criteria of fecal-marker concentrations for successful treatment remain unsettled, requiring studies larger than the present one.

Studies showing calprotectin to predict relapse in clinically inactive IBD have used various cutoff concentrations (250, 150, 130 μg/g) (Tibble *et al* 2000b, Costa *et al* 2005, D'Inca *et al* 2008). Although in Tibble and coworker's trial (2000b) a 50 mg/l cutoff (new assay: 250 μg/g) predicted relapse in both UC and CD, the trial by Costa and coworkers (2005) failed to predict relapse in CD (cutoff for calprotectin >150 μg/g), probably due to their low number of patients with colonic CD. In the latest study, a 130 μg/g cutoff seemed to predict relapse in UC or in colonic CD (D'Inca *et al* 2008). No corresponding data on lactoferrin are available.

IL-17 seems to augment TNFα-induced inflammatory responses, and interactions of TNFα and IL-17 potently mobilize neutrophils (Andoh *et al* 2008). In Study IV, we failed—probably due to a low number of patients—to detect during anti-TNFα therapy a significant correlation with IL-17-positive T cells and neutrophil-derived marker calprotectin. The therapy, however, reduced the mucosal IL-17/CD4 cell ratio associated with endoscopically confirmed mucosal healing. Furthermore, the treatment was reflected as a decreased IL-17/Foxp3 cell ratio, which may indicate an improved balance between effector and regulatory T cells as a consequence of treatment. Although these results may be biased by our small number of patients and sampling error, it seems that TNFα-blocking agents failed to normalize the mucosal IL-17 or IFN-γ upregulation characteristic of CD (Hölttä *et al* 2008). This is in keeping with what is known from clinics: TNF-blocking

agents (or any other known therapy at present) do not cure the disease even though they heal the mucosa. Relapse is very probable after discontinuation of the drug. Although mucosal healing is difficult to achieve, it should become the target of CD treatment. Rutgeerts, Vermeire, and Van Assche have recently concluded:
"The future starts now, and we should raise the bar for our treatment goals as listed below: induction and maintenance of remission without steroids, complete healing of the intestinal and colonic mucosa, avoidance of complications and surgeries, avoidance of cancer, and avoidance of mortality" (Rutgeerts *et al* 2007). For detection of mucosal healing, a need exists in clinical practice and research for surrogate markers such as calprotectin and lactoferrin.

Conclusions

Crohn´s disease is a lifelong chronic disease with a relapsing course. Symptoms sometimes fail to correlate with intestinal inflammation, and repeated examinations are needed to detect mucosal inflammation. In CD, our study showed that fecal calprotectin and lactoferrin correlate closely with the validated endoscopic scores. These markers were significantly higher in endoscopically or clinically active than in inactive disease. A cutoff concentration of 200 μg/g for calprotectin and of 10 μg/g for lactoferrin were specific in detecting endoscopically active disease, but sensitivity was lower. Accordingly, a cutoff concentration of 1000 μg/g for calprotectin and of 50 μg/g for lactoferrin for endoscopically moderately or severely active disease were highly specific.

Fecal markers correlated also with histologic scores of CD in ileocolonic or colonic disease. A normal calprotectin or lactoferrin value was a useful surrogate marker of histologically inactive disease. During anti-TNFα therapy, changes in fecal-marker concentrations failed, however, to correlate with changes in histologic scores.

Fecal calprotectin and lactoferrin were significantly higher in endoscopically active ileocolonic or colonic CD than in inactive disease. Both fecal markers were also higher in active ileocolonic or colonic CD than in active ileal disease. Furthermore, fecal markers failed to correlate with ileal endoscopic or histologic activity in ileal disease. In ileal disease with limited extension, fecal markers may thus not be as good surrogate markers of disease activity as in other CD locations.

Fecal markers correlate more strongly with endoscopic findings than does the CRP, widely used in follow-up of CD patiens. Measurements of either calprotectin or lactoferrin in follow-up of CD patients could be included in everyday clinical work.

During anti-TNFα therapy, both fecal markers decreased significantly and correlated with changes in endoscopic score. Furthermore, in patients achieving endoscopic remission, fecal markers normalized. Based on this study, fecal neutrophil-derived proteins serve well as surrogate markers of mucosal healing and are objective measures of disease activity. Serial fecal-marker measurements during therapy may replace endoscopic follow-up.

During anti-TNFα therapy, mucosal IL-17/CD4 and IL-17/Foxp3 ratios decreased, possibly indicating an improved balance between effector and regulatory T cells.

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