FECAL CALPROTECTIN

The usefulness in special clinical situations and issues on the sampling procedure

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ABSTRACT

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Ulcerative colitis and Crohn's disease are chronic inflammatory bowel diseases (IBD) of unknown etiology. In recent years, mucosal healing has emerged as the goal for therapy to achieve long-term remission and to change the natural course of IBD. Thus, it is essential to monitor thoroughly the disease activity. Fecal calprotectin is the best available biomarker of disease activity in IBD.
The overall aim of this thesis was to study the clinical usefulness of fecal calprotectin. Four different patient cohorts were investigated.

For patients with active ulcerative colitis, the fecal calprotectin levels varied considerably, even over a single day, and the variability was considered to be clinically important in up to one-third of the patients. However, the longer the time period between bowel movements, the higher were the concentrations of calprotectin. To reduce both the impact of the variability and the risk of false low calprotectin values, samples should be obtained from the first stool passed in the morning. In stool samples stored at room temperature, the concentrations of calprotectin were stable for 3 days, while the levels decreased significantly after 7 days. In a questionnaire, the patients declared that they did not find it burdensome to obtain stool samples, although suitable equipment was considered desirable.

The levels of fecal calprotectin did not distinguish between patients with endoscopic recurrence 1 year after ileocaecal resection for Crohn's disease and those without. However, in patients with low calprotectin values, endoscopic remission was commonly noted, suggesting that a colonoscopy might be avoided in these cases.

In the group of patients with quiescent ulcerative colitis, dose escalation of 5-aminosalicylic acid (5-ASA) in those patients identified with increased levels of calprotectin significantly reduced the relapse rate. However, the overall relapse rate of the intervention group was not significantly lower than that of the control group.

At cut-off values for calprotectin of 169 µg/g and 262 µg/g, the clinical course in patients with newly diagnosed ulcerative colitis could be predicted with good specificity and moderate sensitivity, for 1 and 3 years, respectively.

Conclusions: These results facilitate standardization of the stool sampling procedure, which is necessary to improve the accuracy of this biomarker. Furthermore, fecal calprotectin might be used to select patients for ileocolonoscopy 1 year after ileocaecal resection for Crohn's disease. To treat patients with IBD in clinical remission, but with increased values of calprotectin suggesting subclinical disease activity, brings a new dimension to IBD care. In this context, dose escalation of 5-ASA may be appropriate in patients with ulcerative colitis. This therapeutic concept should be tested also in patients with new onset of ulcerative colitis.

Keywords: Inflammatory bowel disease; ulcerative colitis; Crohn's disease; fecal biomarker; calprotectin; 5-aminosalicylic acid; ileocaecal resection; colonoscopy.

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LIST OF PAPERS

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals:

I. **The intra-individual variability of faecal calprotectin: A prospective study in patients with active ulcerative colitis.**
Anders Lasson, Per-Ove Stotzer, Lena Öhman, Stefan Isaksson, Maria Sapnara, Hans Strid
J Crohn's Colitis 2014 Jul 5. pii: S1873-9946(14) [Epub ahead of print]

II. **Fecal calprotectin one year after ileocaecal resection for Crohn's disease — A comparison with findings at ileocolonoscopy.**
Anders Lasson, Hans Strid, Lena Öhman, Stefan Isaksson, Mikael Olsson, Britt Rydström, Kjell-Arne Ung, Per-Ove Stotzer
J Crohn's Colitis (2014) 8, 789–795

III. **Pharmacological intervention based on fecal calprotectin levels in patients with ulcerative colitis at high risk of a relapse: A prospective, randomized, controlled study.**
Anders Lasson, Lena Öhman, Per-Ove Stotzer, Stefan Isaksson, Otto Überbacher, Kjell-Arne Ung, Hans Strid
Submitted for publication

IV. **Fecal calprotectin levels predict the clinical course in patients with new onset of ulcerative colitis.**
Anders Lasson, Magnus Simrén, Per-Ove Stotzer, Stefan Isaksson, Lena Öhman, Hans Strid
Inflamm Bowel Dis 2013;19:576–581

CONTENTS

ABBREVIATIONS

5-ASA	5-aminosalicylic acid
AUC	Area under the curve
CDAI	Crohn's disease activity index
CDEIS	Crohn's disease endoscopic index of severity
CF	Cystic fibrosis
CI	Confidence interval
CNS	Central nervous system
CRP	C-reactive protein
CV	Coefficient of variation
DAI	Disease activity index
ESR	Erythrocyte sedimentation rate
ELISA	Enzyme-linked immunosorbent assay
HBI	Harvey Bradshaw index
IBD	Inflammatory bowel disease
IBDU	Inflammatory bowel disease unclassified
IBS	Irritable bowel syndrome
IBSEN	Inflammatory bowel disease in south-eastern Norway
ICC	Intraclass correlation coefficient
IQR	Inter-quartile range
MRI	Magnetic resonance imaging
MRP	Myelomonocyte related protein
NPV	Negative predictive value
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
PPV	Positive predictive value
SD	Standard deviation
SES-CD	Simple endoscopic score for Crohn's disease
TNF	Tumor necrosis factor

1 INTRODUCTION

1.1 Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) represent a group of chronic disorders of the intestines with unknown etiology. *Ulcerative colitis* and *Crohn's disease* are the two major diseases of this group, and they will be discussed separately in the following sections. These two disorders have many features in common, including a presumed etiology that involves interactions between environmental factors, the intestinal microbiota, the host immune system, and predisposing genetic factors. Furthermore, as the clinical presentations of these two diseases are sometimes similar they can be difficult to differentiate. In 1978 the term *Indeterminate colitis* was introduced for such cases, and since 2005 the term *Inflammatory Bowel Disease Unclassified* (IBDU) has been established[1, 2]. The frequency of this entity is about 10% of IBD patients, and appears more frequently in pediatric patients than in adult patients[3].

In 1976, the Swedish pathologist Clas Lindström first described a new entity of chronic colitis, termed *Collagenous colitis,* and in 1989, *Lymphocytic colitis* was described for a similar disorder [4, 5]. The term Microscopic colitis has been widely used as an umbrella term for these two disorders with similar clinicopathologic features, characterized by chronic diarrhea, typically in middle-aged and elderly women, and with normal, or almost normal appearance of the colonic mucosa upon endoscopy. Diagnosis of such patients relies on examinations of biopsies taken from the colon[6].

In summary, ulcerative colitis, Crohn's disease and the unclassified IBDU traditionally constitute the overall group of IBD. In recent years, even the two entities of microscopic colitis, collagenous colitis and lymphocytic colitis, have been commonly included with the IBD disorders. The incidence of IBD has been increasing worldwide, although it is highest in Europe and North America. The highest incidences reported for ulcerative colitis and Crohn's disease are $24.8/10^5$ (Finland) and $20.2/10^5$ (North America), respectively[7, 8]. In Sweden, the annual incidences of ulcerative colitis and Crohn's disease are reported as $17.5\text{-}20.0/10^5$ and $8.3\text{-}9.9/10^5$ persons, respectively and about $5\text{-}7/10^5$ persons for

each of collagenous colitis and lymphocytic colitis [9-14]. Thus, the overall prevalence of IBD in Sweden is close to 1%[15].

1.1.1 Ulcerative Colitis

Ulcerative colitis is the most prevalent of the IBD disorders. In a study from Örebro, the prevalence in 1987 was 234/10^5, and quite recently the prevalence was estimated as 350/10^5 persons in a Swedish nationwide register-based study[16, 17]. While the incidence of ulcerative colitis is increasing worldwide, the reason for this is unknown. In epidemiologic studies repeated in the same catchment area, a trend of increasing incidence, even during the last decades, has been reported[7, 9, 10]. In Europe, the prevalence of ulcerative colitis varies in the range of 2.4-350/10^5 persons, with the highest numbers reported from Northern Europe[17, 18]. The formerly noted North-South gradient of ulcerative colitis distribution seems to have changed in recent years to an East-West gradient within Europe[19].

Disease onset is typically in the 15-40-year age group, even though ulcerative colitis can occur at any age[20]. Males and females are affected approximately equally[21, 22]. The disease onset is usually insidious, although it can start with an acute severe attack. A loose stool, that usually contains blood and a mucopurulent exudate is the key symptom, and the disease is often associated with rectal urgency and abdominal pain or discomfort. In severe attacks, weight loss and fever are frequently seen. In about 10% of cases extraintestinal manifestations, such as arthritis, pelvospondylitis, erythema nodosum and episcleritis, are observed[23].

In patients with symptoms suggestive of ulcerative colitis, an endoscopic assessment must be carried out. Preferably, an ileocolonoscopy is performed and biopsies are obtained for histopathologic evaluation to establish the diagnosis. Other types of colitis, primarily infectious colitis, have to be excluded. Thus, the diagnosis is made based on the clinical presentation, endoscopic observations and the results of histologic testing.

Ulcerative colitis is characterized by a diffuse continuous inflammation, which typically starts in the rectum and extends proximally in the colon. The colonic extension of the disease is classified based on the endoscopic evaluation. Thus,

according to the Montreal classification, the disease is classified as: proctitis (limited to the rectum, extending ≤ 15 cm); left-sided colitis (> 15 cm, but not extending beyond the splenic flexure); and extensive colitis (extension proximal to the splenic flexure)[2]. At presentation, patients with ulcerative colitis are approximately evenly distributed between these three groups. However, in children extensive colitis is more common[10]. During the clinical course, colonic extension of the disease increases in a substantial proportion of patients[24, 25]. To classify the disease appropriately is clinically very important, since the extent of the disease has implications for the treatment and follow-up.

The clinical course of ulcerative colitis varies with the individual patient. In the IBSEN study, a Norwegian population-based cohort followed over 10 years from disease onset, just over 50% of the patients described a clinical pattern of remission or mild severity of intestinal symptoms after an initial high activity. About one-third of the patients described chronic intermittent symptoms and 6% described a chronic continuous disease pattern[25]. Ten years after disease onset, relapse rates of 67-97% have been described in European studies[25-28]. In most cases, the reason for relapse is unknown. However, several factors that contribute to relapsing disease have been proposed, including smoking history, age, gender, level of education, and use of non-steroidal anti-inflammatory drugs[23, 25, 26, 28]. More recently, complete mucosal healing has emerged as possibly the most important factor for the clinical course and accordingly, this is the goal for modern treatments[29, 30].

Current treatments for ulcerative colitis are mainly pharmacologic, with the aim of inducing and maintaining remission. To choose the appropriate treatment, the severity of the disease and its distribution in the colon has to be considered. To induce remission, 5-aminosalicylate (5-ASA) and corticosteroids are the most frequently used agents. In some patients who are refractory to these drugs or who suffer a severe attack, biological treatment with an anti-tumor necrosis factor (anti-TNF) agent or the recently approved anti-integrin $\alpha_4\beta_7$ antibody vedolizumab, may be appropriate. To maintain remission, 5-ASA, an immunomodulating drug (almost exclusively a thiopurine agent) and an anti-TNF agent can be used either alone or in combination[31].

In those patients who are refractory to medical treatment and in patients who experience complications, a surgical approach, involving colectomy or procto-

colectomy, is recommended. Mortality rates were high before the era of appropriate use of corticosteroids and surgery, especially for patients who suffered their first attack or had severe disease. The decision to proceed to the inevitable colectomy for patients who are unresponsive to medical therapy has had a major impact in reducing mortality[32]. Today mortality rates for patients with ulcerative colitis are no different from those for the general population[33]. The cumulative colectomy rates 10 years after disease onset are approximately 10% in different cohorts, with higher rates reported from Copenhagen[21, 25, 34]. The risk for colectomy is highest in patients who have extensive colitis and during the first years post-diagnosis[25, 35].

Patients with ulcerative colitis, especially those with extensive and chronic active disease, are at increased risk to develop colorectal cancer[36-38]. More recent data indicate that the risk may be lower than previously thought, and in some studies, no increased incidence of colorectal cancer was found[21, 39, 40]. Still, it is recommended that surveillance colonoscopies with biopsies to detect dysplasia, as well as cancer, are conducted on a regular basis[41].

1.1.2 Crohn's disease

Crohn's disease was first described in 1932 by Dr Burril B Crohn in a paper with the title *Regional Ileitis: A Pathologic and Chronic Entity*[42]. A Polish surgeon, Antoni Leśniowski, published reports on this condition at approximately the same time and in some contexts, especially in Polish publications, the disorder is referred to as Leśniowski-Crohn's disease. The designation regional enteritis is still used in the literature.

The highest prevalences of Crohn's disease, at $322/10^5$ and $319/10^5$ persons,, have been reported from a small town in Sicily, Italy and from Nova Scotia in Canada, respectively [43, 44]. However, as is the case for ulcerative colitis, the prevalence of Crohn's disease varies across the world and epidemiologic data for many parts of the world are missing. The prevalence of Crohn's disease is increasing worldwide[8]. In reports from Europe the prevalence varies within the range of $1.5\text{-}322/10^5$ persons, with the highest rates reported from Northern Europe[18]. In a paper published in 1996, Anders Lindgren and colleagues reported a prevalence of $94/10^5$ in Gothenburg, and in more recent publications

the prevalence has been reported to be 190-213 cases per 10^5 inhabitants in Sweden[11, 17, 45].

Crohn's disease is a lifelong disorder, most frequently presenting in late adolescence or early adulthood and it is equally distributed between the sexes[46]. Chronic diarrhea, abdominal pain and weight loss are the most common initial symptoms. Since Crohn's disease can be located anywhere along the gastrointestinal tract, the symptoms reported at presentation can vary. Likewise, the symptoms are influenced by the occurrence of complications, such as strictures, abscesses and fistulas[47]. Extraintestinal manifestations are similar to those mentioned for ulcerative colitis. An increased mortality rate for patients with Crohn's disease has been reported in some studies, although this has not been confirmed by others[48-50].

The diagnosis of Crohn's disease is based on clinical presentation, evaluation of the entire intestinal tract, and histopathologic findings[51]. An ileocolonoscopy is usually performed, to evaluate the colon, and the terminal ileum and to obtain biopsies. The endoscopic features of Crohn's disease are typically discontinuous inflammatory lesions, aphthous ulcers, linear ulcers, cobblestoning, presence of strictures and perianal involvement[41]. Moreover, the small intestine must be examined. In this respect, both magnetic resonance imaging (MRI) and computed tomography enterography have high diagnostic accuracy for Crohn's disease in the small intestine[52]. Transabdominal ultrasound is sometimes an alternative and in selected cases, small bowel capsule enteroscopy is used to complement previous examinations.

Crohn's disease is characterized by the finding of discontinuous transmural granulomatous intestinal inflammation[51]. The transmural nature of the inflammation, which is an important difference between Crohn's disease and ulcerative colitis, is the underlying cause of many of the complications seen with Crohn's disease. Similar to ulcerative colitis, Crohn's disease has been classified in the Montreal document, which is used in clinical as well as research applications[2]. The classification relies on three different categories: age at diagnosis (< 16 y, 17-40 y, > 40 years); disease location (ileal, colonic, ileocolonic, isolated upper disease); and disease behavior (non-stricturing non-penetrating, stricturing, penetrating). In population-based studies, approximately one-third of the patients had ileitis, colitis or ileocolitis, respectively, at the time

of diagnosis, and only a small minority had isolated upper disease at presentation. A stricturing or penetrating disease behavior was noted in up to one-third of the patients at diagnosis[19, 53].

In studies conducted at referral centers, as well as in population-based studies changes in disease behavior over time have been reported[54, 55]. Thus, the percentage of patients with stricturing and penetrating disease increases over time, whereas the inflammatory burden decreases over time. The need for surgery increases over time and within 10 years 35-50% of the patients had undergone intestinal resection[53, 54, 56, 57]. Disease phenotype has been reported to be associated with the need for surgery. Risk factors, such as terminal ileal location, upper gastrointestinal disease, stricturing or penetrating disease behavior, and young age at diagnosis, have been identified[54, 57]. However, the surgical rates are falling[21]. It is possible that modern therapy, which includes immunomodulators and biologic agents, have changed the natural course of Crohn's disease, although some reports suggest that surgical rates were falling already prior to the advent of biologic therapies[21, 58, 59].

Treatment of Crohn's disease often requires a multidisciplinary approach, combining the skills of gastroenterologists and surgeons to ensure success. As is the case for ulcerative colitis, the etiology of Crohn's disease is unknown and accordingly, causal therapy is not available. The activity, location, and behavior of the disease must be taken into account when therapy is being planned. To give a simplified picture of the treatment, medical therapy is used in inflammatory active disease and the main indications for surgery are complications. Corticosteroids, immunomodulatory drugs (methotrexate and thiopurines), and biologic therapy (mainly anti-TNF agents), used alone or in combination are the most commonly used agents for the treatment of patients with Crohn's disease. In some cases, antibiotics or sulfasalazine (in cases of mildly active colonic disease) can be an option[60]. Examples of surgical interventions are: intestinal resections; stricturoplasty of the small intestine; drainage of abscesses; and cleavage of fistulas. After resection, the risk of disease recurrence has to be considered, and in most patients, post-surgical maintenance treatment is recommended[61].

An increased risk of intestinal cancer, particularly in the small intestine, has been reported in Crohn's disease[62]. However, as the risk of small bowel cancer

in the background population is very low, the clinical consequence of the increased risk in patients with Crohn's disease is not so serious. Although the magnitude of the colorectal cancer risk in Crohn colitis remains a matter of debate, as in the case of ulcerative colitis, surveillance colonoscopy is recommended[39, 41].

1.2 Histopathology in Ulcerative colitis and Crohn's disease

To understand the roles of fecal biomarkers as surrogate markers of disease activity in IBD, a brief overview of the histopathology is appropriate.

The diagnosis of IBD is established by considering several clinical findings, and the histologic examination is a key step towards a correct diagnosis[23, 47]. The samples used in the histologic examination are obtained at endoscopy and from surgically resected specimens.

In active ulcerative colitis and Crohn's disease, the abundant mucosal infiltration of neutrophilic granulocytes is characteristic. The strongest histologic predictor of IBD in patients who have suffered a first attack of the disease is basal plasmacytosis[63]. Typical findings in cases of ulcerative colitis are: crypt atrophy; crypt distortion; superficial erosions; and infiltration of the surface epithelium by neutrophils. In the early stages of the disease, not all of these features are present. The characteristic findings in cases of long-standing disease are: crypt architectural distortion; a diffuse transmucosal inflammatory cell infiltrate; cryptitis; and crypt abscesses[64].

Characteristic of Crohn's disease, is a focal, discontinuous chronic inflammation with plasma cells and lymphocytes not only in the superficial layers, but also in the lamina propria. Furthermore, focal crypt irregularities and granulomas are accepted microscopic features. In the small intestine, an irregular villus architecture can be found. Since Crohn's disease is a discontinuous inflammation with normal mucosa located between inflamed or ulcerated mucosa, the risk for sampling error is obvious. Thus, not all of the classical histopathologic features may be present in the available biopsies. Examples of additional useful features are focal cryptitis and aphtoid ulcers[64].

1.3 Assessment of disease activity in IBD

There is no single test or examination that fulfills all the diagnostic requirements of the clinicians who are treating IBD patients. A combination of reported symptoms, clinical examination, endoscopy, radiology and laboratory markers will remain the basis for assessments of disease activity in the foreseeable future. However, the importance of a reliable, simple, non-invasive or only minimally invasive, highly sensitive and reproducible marker of disease activity cannot be overstated. The goal of modern treatment with immunomodulators and biologic agents is mucosal healing with consequent improvement of the natural course of IBD. To achieve this, disease monitoring on a regular basis is mandatory[65].

Currently, endoscopy, particularly ileocolonoscopy, is the 'Gold standard' for assessing disease activity in patients with IBD, offering the possibility for direct visualization of the mucosa and biopsy sampling for histopathologic evaluation[23, 47]. However, ileocolonoscopy is an invasive procedure with certain shortcomings. The invasive nature of the technique carries risks for complications and patient discomfort, as does the inevitable bowel preparation. Ileocolonoscopy is not always complete, and in patients with Crohn's disease in the small bowel it is not accurate. To a certain extent, the mucosal inspection is subjective, with room for individual interpretations. Moreover, ileocolonoscopy is expensive and is not always readily available. Thus, a simpler method for disease monitoring in daily practice is desirable.

1.3.1 Serologic markers

Several laboratory markers have been studied in IBD. The most widely used and most intensively evaluated tests are the C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR)[65]. Other commonly used markers include white blood cell counts, platelet counts, and the levels of albumin and orosomucoid. These markers all have drawbacks in terms of being influenced by other inflammatory activities, stress, ongoing treatment for IBD, nutritional status, and long half-life in serum. Moreover, they have not shown any advantages over CRP in the diagnosis and monitoring of disease activity in patients with IBD[66].

CRP is an acute-phase protein that is principally synthesized by hepatocytes. In situations of systemic inflammation, the production of CRP is rapidly increased as a response to inflammatory cytokines, mainly Interleukin 6. The short half-life (19 hours) and easy, inexpensive laboratory analysis make CRP a reliable and simple marker to use in daily practice[67]. Measurement of CRP can be used at diagnosis of IBD, and facilitates the differentiation of IBD from functional bowel disorders[68]. However, in the IBSEN study a majority of the patients with ulcerative colitis and 25% of the patients with Crohn's disease had a normal level of CRP (≤ 10 mg/l) at diagnosis[69]. An association between CRP and endoscopic activity in IBD has been confirmed, as has the usefulness of CRP for monitoring responses to therapy[70, 71]. Overall, patients with Crohn's disease have higher CRP production than patients with ulcerative colitis, and CRP is a much more reliable marker of disease activity in Crohn's disease than in ulcerative colitis[65, 69, 70]. However, CRP is, like all other serologic markers, at best a non-specific marker of systemic inflammation.

1.3.2 Radiolabeling techniques

To overcome the non-specificity of serologic markers, the techniques of using intestinal permeability to assess the small intestine and fecal excretion or scanning of ^{111}In-labeled granulocytes have been adopted as non-invasive tests for IBD. The former method has been used for the identification of patients with small bowel Crohn's disease and for the follow-up of therapy[72]. The fecal excretion of ^{111}In-labeled granulocytes has been regarded as the 'Gold standard' of disease activity in ulcerative colitis and Crohn's disease[73]. However, as these methods are cumbersome, expensive, and involves exposure to radiation, they have been used almost exclusively for research purposes.

1.3.3 Clinical and endoscopic disease activity indices

To meet the demands for numerical measurements of disease activity in clinical trials, several clinical activity indices have been established. Most of these have been used exclusively in clinical trials and actually, almost all of our modern treatment strategies for IBD rely on studies in which a change in a defined index is the primary endpoint. The numerous activity indices used in clinical trials

have been presented in two review articles[74 75]. In all, 12 clinical activity indices and 11 endoscopic indices are presented, and additional combined clinical and endoscopic, as well as modifications of the original indices are reviewed. Some of these merit discussion in greater detail.

The most widely used index in clinical practice is the Truelove and Witts Severity Index for ulcerative colitis[76]. This index has been particularly useful in identifying patients with a severe attack, defined as: bloody stool frequency ≥ 6/day, and in addition at least one of the following clinical parameters: tachycardia (> 90 bpm); fever (> 37.8°C); anemia (haemoglobin < 105 g/l); elevated ESR (> 30 mm); or CRP (> 30 mg/l). This index is not suitable for measuring changes in disease activity, although it can be used to classify broadly the patients, for example to identify patients who require admission to the hospital.

Another commonly used index in ulcerative colitis is the Mayo Score (also known as the Mayo Clinic Score and the Disease Activity Index, DAI)[77]. Details of this index are presented in the *Methods* section.

For cases of Crohn's disease, the Crohn's Disease Activity Index (CDAI) is by far the most frequently used instrument[78]. In this scoring system eight items, which combine subjective symptoms (i.e., the number of liquid stools, abdominal pain, and overall well-being) with objective findings upon examination and laboratory test results (i.e., the need for antidiarrheal drugs, extraintestinal complications, the presence of an abdominal mass, haematocrit, and weight), are weighed together. The scores are based on 1 week of symptoms and range from 0 to approximately 600. In clinical trials, the cut-off for remission is usually set at 150 points. However, the CDAI has been criticized with respect to interobserver variability, a poor correlation with endoscopic disease activity, the strong impact of subjective symptoms, the lack of a parameter for inflammatory activity, and finally, the complexity of the instrument[74, 79].

The Harvey Bradshaw Index (HBI) is a simplified version of the CDAI[80]. These two indices correlate with each other. The main advantage of the HBI is that it only contains clinical parameters, making it easier to use. However, the HBI lacks markers of inflammatory activity (*cf.* Table 4, in the *Methods* section).

To assess endoscopic disease activity in patients with ulcerative colitis, the endoscopic subscore of the Mayo score is frequently used. In Crohn's disease the Crohn's Disease Endoscopic Index of Severity (CDEIS) and the simplified alternative version, the Simple Endoscopic Score for Crohn's Disease (SES-CD), are the two validated instruments[81, 82]. Owing to their levels of complexity, these indices have to date been used mainly in clinical trials.
To assess Crohn's disease in the postoperative setting and to predict the clinical course after surgery, a five-graded scoring system has been presented by Rutgeerts' et al[83]. The scoring system is presented in detail in the *Methods* section.
Indices for histologic assessment of disease activity have also been constructed, but these will not be discussed here[74].

1.3.4 Imaging techniques

Computed tomography and magnetic resonance enterography (MRI) and transabdominal ultrasound are the more advanced techniques for disease activity assessment. A good correlation between disease activity found at endoscopy and MRI has been reported[84]. Scoring systems for disease activity assessed with MRI have been developed, and they correlate with fecal calprotectin concentrations[85]. The MRI is non-invasive, reproducible, does not involve ionizing radiation, and provides the opportunity to examine the entire gastrointestinal tract, including potential complications, such as fistulas in Crohn's disease. However, the technique is costly and is not readily available. Transabdominal ultrasound is a non-invasive, radiation-free method with potential to become easily available. It is an attractive modality for the evaluation of patients, mainly those with Crohn's disease. However, this method is still under evaluation and has several shortcomings. For example, the accuracy of the method has been called into question and is highly investigator-dependent[86].

In summary, the wide spectrum of biomarkers, clinical indices, and imaging modalities currently in use is probably an indication that we still don´t have the ideal marker to assess readily disease activity in patients with IBD. However, the analysis of neutrophil-derived proteins in fecal samples appears to be a

novel, and promising contribution to this field and will be discussed in the next section.

1.4 Fecal biomarkers

The concept of using the concentrations of neutrophil-derived proteins in the feces of patients with IBD as a marker of disease activity is brilliant. The number of inflammatory cells in the intestinal mucosa reflects the level of inflammatory activity, as shown, for instance, with techniques that use ^{111}In-labeled granulocytes[73]. Analysis of the feces for these proteins derived from inflammatory cells, mainly granulocytes, provides the opportunity to assess both easily and specifically the inflammatory burden in the gastrointestinal tract. However, in this context, it is important to stress that none of the stool markers are specific for IBD, but they are rather markers of infiltration into the mucosa by inflammatory cells[87].

While many of these neutrophil-derived proteins have potential to be used as surrogate markers of intestinal inflammation, disease monitoring with calprotectin has become the most widespread in clinical practice. Furthermore, no other stool marker has shown significantly better utility in the management of IBD patients. A brief summary of two other thoroughly investigated fecal markers, namely lactoferrin and S100A12, will be given.

Lactoferrin is a glycoprotein that is stored in the secondary granules of neutrophils, although it is also present in other cell types, such as epithelial cells. It has both antibacterial and antifungal properties, is fairly stable at room temperature, and is easily quantified by ELISA[88]. In pooled data from 1001 patients, the estimated sensitivity and specificity were 80% and 82%, respectively, to identify correctly patients with IBD[89]. The level of lactoferrin correlates with the endoscopic grade of inflammation in ulcerative colitis and in Crohn's disease[90, 91]. In most studies, the performances of lactoferrin and calprotectin as biomarkers of disease activity are similar[90].

S100A12 (known as calgranulin C) is, like calprotectin, a member of the S100-protein family with pro-inflammatory properties. It is almost exclusively restricted to the granulocyte cytosol, so theoretically it has properties that make it more advantageous than other available fecal markers[87]. In a study conducted

by Kaiser et al, S100A12 performed even better than calprotectin[92]. While it is still a promising biomarker, S100A12 has to date not been studied as extensively as calprotectin in different clinical situations, and further studies are needed to confirm the initial encouraging results.

Several other fecal biomarkers, such as lysozyme, polymorphonuclear neutrophil-elastase, M2-pyruvate kinase, metalloproteinases, chromogranins, and eosinophil protein X, have been evaluated in patients with IBD. However none of these have performed better than calprotectin, and many of them have drawbacks, such as insufficient stability[93].

1.5 Calprotectin

Calprotectin was first described by Magne Fagerhol and colleagues in 1980[94]. Instead of using isotope-labeled leukocytes to describe the turnover of leukocytes, they suggested that monitoring the proteins released from leukocytes would be a simple and clinically useful strategy. Consequently, they described the quantification of a leukocyte-derived protein, which was called the L1-protein (Leukocyte protein candidate 1).

High plasma levels of the L1-protein, as compared to the levels in healthy controls, were reported in patients with malignant diseases and bacterial infections[94, 95]. The authors concluded that the L1-protein could be of interest in clinical practice, and they even showed that it was more sensitive than the erythrocyte sedimentation rate (ESR) in these patients[94].

The name calprotectin was proposed in 1990, as it had been shown that this calcium-binding protein had antimicrobial effects[96]. During the 1980's, other researchers identified the cystic fibrosis antigen, also called calgranulin, and the myelomonocyte-related proteins, MRP-8 and MRP-14[97-100]. In 1988, Andersson et al showed that the amino acid sequences and immunohistochemical staining patterns of MRP-8 and CF-antigen were identical to those of the light chain in the L1-complex and that MRP-14 was identical to the heavy chain of that complex[101]. While all these different names are used in the literature, they refer to the same protein, calprotectin.

Calprotectin has been further characterized as a 36.5 kDa protein that consists of two heavy and one light chain[102]. Calprotectin is one of more than 20 proteins in the so-called S-100 family. These proteins were named because of their solubility in 100% ammonium sulfate solution[103]. They are all calcium-binding proteins with various intra- and extracellular regulatory properties[104]. Calprotectin is made up of the S100A8/S100A9 complex, and is primarily active extracellularly. The heterodimeric S100A8/S100A9 complex binds six calcium atoms and can also bind zinc and manganese ions. This is considered to be one of the several mechanisms underlying the antimicrobial effects of calprotectin. Calprotectin is heat-resistant and resistant to proteolysis in the presence of calcium[97].

Calprotectin is found mainly in neutrophilic granulocytes, in which it accounts for about 5% of the total protein content and up to 60% of the cytosolic proteins[94, 97]. Neutrophilic granulocytes and monocytes originate from the same progenitor cell[105]. This explains why they express many similar cytoplasmic products. Consequently, calprotectin has also been found in monocytes and in macrophages, albeit to substantially lesser extents than in granulocytes. Furthermore, calprotectin is expressed in the cells of the mucosal and squamous epithelia and in pancreatic cell lines[106,107].

During the inflammatory process, the S100A8/S100A9 complex (calprotectin) is released from activated phagocytes and epithelial cells, and exhibits pro-inflammatory properties[87]. In non-infectious situations, as in IBD, the initial trigger for this inflammatory process is unknown. Epithelial cells and the innate immune system are activated, leading to the active secretion of cytosolic proteins, cytokines (including TNF), and chemokines from monocytes and granulocytes. The activation of epithelial cells is the first source of calprotectin secretion into the intestinal lumen. Calprotectin molecules in the mucosa bind to endothelial cells, resulting in the recruitment of more leukocytes, thereby accelerating the inflammatory cascade[108, 109]. As this process is amplified, a delicate interaction between the molecules from phagocytic cells and different cell types, especially epithelial cells, leads to the transmigration of neutrophils and subsequently, mucosal damage. Most of the calprotectin in the stool originates from granulocytes as they are activated and subsequently undergo necrosis, releasing the cytosolic content into the intestinal lumen[87, 110].

The antimicrobial activity of calprotectin was first reported by Steinbakk et al, as antifungal and antibacterial activities[96]. Inhibition of metalloproteinases by calprotectin has been reported[111], and as previously mentioned, chelation with zinc and manganese ions by calprotectin is proposed to inhibit microbial proliferation, as these metals are of vital importance for bacterial growth[112-114]. Calprotectin has with good reason been referred to as a physiologic antibiotic agent[115]. Furthermore, calprotectin induces the apoptosis both in malignant and non-malignant cell-lines[116]. The potential roles of calprotectin in tumor pathogenesis, tumor growth and even atherosclerosis are examples of new and interesting areas of research[117-119]. Moreover, various intracellular functions of calprotectin have been proposed[104]. Thus, calprotectin is an abundant ubiquitous protein, and it is obvious that we have a lot more to learn about its functions in various biological situations and in the subsequent evaluations of its usefulness in clinical science.

Initially, calprotectin was analyzed mainly in the plasma, for instance in patients with rheumatoid arthritis[120-122]. However, measurement of the plasma concentration of calprotectin has not yet made a breakthrough in the clinic[123]. The same applies to other areas of clinical medicine in which calprotectin has been evaluated, e.g., infectious diseases, malignant diseases, cystic fibrosis, urinary tract diseases, CNS inflammatory diseases and myocardial infarction. However, there is one important exception, whereby measurement of calprotectin has been established as the best marker of disease activity, namely the measurement of calprotectin in the feces of patients with IBD[124].

1.6 Fecal calprotectin in clinical practice

In 1992 Arne Røseth and coworkers published the first paper describing the measurement of calprotectin levels in feces[125]. In this pioneering study and in the subsequent publications from the same group, fundamental data for continued clinical research were established. Practical issues, such as the stability at room temperature, the correlation between using a spot sample of feces and a complete stool collection, the correlation with the fecal excretion of ^{111}In-labeled granulocytes, the elevated levels of calprotectin in bowel diseases, especially IBD, and the correlation between the calprotectin levels and the endoscopic and histologic disease activities in ulcerative colitis were

addressed[125-127]. Subsequently, numerous studies have confirmed the usefulness of fecal calprotectin, primarily in the diagnosis of IBD, to assess disease activity in IBD and to predict the disease course in patients with IBD.

1.6.1 Fecal calprotectin as a diagnostic tool

The symptoms of IBD are not specific, and many of the patients who present with abdominal pain or diarrhea will have a normal colonoscopy and several of these patients will suffer from irritable bowel disease (IBS)[128]. Conversely, IBS-like symptoms are frequently present in patients with IBD[129, 130]. Thus, a marker for selecting symptomatic patients for further investigation, and in particular, to distinguish patients with IBD from those with IBS is warranted and, as shown in a quite recent study, is cost-effective[131]. In studies that have addressed this issue, the sensitivity and specificity of fecal calprotectin to diagnose correctly IBD in adults during a subsequent colonoscopy have been calculated as 63-100% (median 83%) and 74-100% (median 90%), respectively[90,92,132-139]. Similar results have been reported from pediatric studies[140]. The cut-off values for calprotectin in these studies ranged from 25 to 170 µg/g, but were commonly in the range of 50 - 100 µg/g. A few meta-analyses have been conducted. As they have attracted much attention, they will be presented briefly.

The diagnostic precision of fecal calprotectin for IBD in nine prospective studies was analyzed in a meta-analysis by von Roon et al[141]. In a pooled analysis, the sensitivity and specificity for fecal calprotectin to identify correctly patients with IBD were 89% and 81%, respectively, with 50 µg/g as the cut-off for calprotectin, and were even higher when a cut-off of 100 µg/g was used[141]. In another meta-analysis conducted by Henderson et al eight pediatric reports comprising in total 715 patients with suspected IBD were included[142]. The pooled sensitivity and specificity values for the diagnostic utility of fecal calprotectin were 98% and 68%, respectively.

In a meta-analysis from the Netherlands, van Rheenen et al included 13 high-quality studies, including only patients (n=1041) with suspected IBD[143]. In most of these studies the cut-off value was 50 µg/g. For adults, the pooled sensitivity and specificity were 93% and 96%, respectively, and for children 92% and 76%, respectively. Furthermore, using fecal calprotectin as a screening method before

colonoscopy would result in a 67% reduction in the number of adults requiring endoscopy. However, 6% of the patients would have a false-negative test, delaying accurate diagnosis of those patients.

All these studies were performed in referral centers, with a risk of bias in relation to patient selection. A recently published study that used fecal calprotectin testing in a primary care setting confirms the utility of fecal calprotectin[144]. However, a higher cut-off value was suggested to improve the positive predictive value in this population with a low frequency of organic diseases. Thus, increasing the cut-off value from 50 to 150 µg/g would reduce the negative predictive value from 98% to 97%, whereas it would increase the positive predictive value from 28% to 71%[144].

In a study with over 600 patients, Tibble et al evaluated the accuracies of several markers, including fecal calprotectin, in distinguishing organic from non-organic intestinal disease[145]. The sensitivity and specificity of calprotectin for identifying organic disease were 89% and 79%, respectively, and the Rome criteria for IBS performed at a similar level. The authors propose combined testing for patients who have positive Rome criteria suggestive of IBS. A negative test result for fecal calprotectin in those patients would confirm the diagnosis of IBS with high probability and would provide reassurance to the physicians that the clinical diagnosis of IBS is correct. This approach would be very useful in clinical practice.

1.6.2 Fecal calprotectin to assess disease activity in IBD

Accurate assessment of inflammatory activity in IBD patients is increasingly important, as mucosal healing has emerged as an important goal for therapies to maintain remission and is probably crucial to change the natural course of IBD[146]. The difficulty to distinguish functional bowel symptoms from symptoms related to active inflammation has been mentioned. Endoscopy is the best way to assess disease activity in IBD. However, endoscopic evaluation is burdensome for both the patient and the clinic. Instead, the measurement of fecal calprotectin levels has emerged as a simple way to monitor disease activity. To evaluate the ability of fecal calprotectin to reflect accurately the disease activity, a correlation has been made between the level of mucosal inflammation detected upon

ileocolonoscopy and the levels of calprotectin[91, 126]. This is discussed in later sections, but it can be stated briefly that the levels of calprotectin correlate significantly with the endoscopic assessments of both Crohn's disease and ulcerative colitis. Furthermore, none of the other evaluated biochemical parameters or clinical indices performed better than calprotectin.

Symptoms of IBS-type are observed in up to 50% of patients who have IBD in remission[129, 147]. In several studies, fecal calprotectin has been used to determine whether subclinical inflammation explains this high prevalence of IBS-like symptoms[130, 148-150]. Although the results are conflicting, it seems that sub-clinical inflammation partly explains this phenomenon[148]. Accordingly, fecal calprotectin has a role to play in distinguishing IBS-like symptoms caused by inflammatory activity from the non-inflammatory symptoms in patients with IBD.

1.6.3 Fecal calprotectin to predict the disease course

In 2000 Tibble et al evaluated fecal calprotectin as a surrogate marker to predict the clinical course in patients with IBD[151]. Patients with IBD in clinical remission were included, and the level of fecal calprotectin was determined at inclusion, and the clinical course was evaluated over a period of 1 year. In summary, patients who had a calprotectin value > 50 mg/L (=250 µg/g) at inclusion were at considerable risk of a relapse during the following year, whereas patients with a calprotectin level < 50 mg/L had a good chance to maintain remission. Since this first study was published, several other papers have been published on this topic and a meta-analysis was reported recently[152]. The results have been consistent. This will be discussed in more detail later.

The usefulness of fecal calprotectin to predict the outcome of anti-TNF therapy in patients with IBD has also been assessed. Fecal calprotectin has proven useful for predicting remission during induction therapy, as well as during 1 year of follow-up[153, 154]. Therefore, patients who have a good clinical response and a normalized calprotectin value during induction therapy with an anti-TNF agent will have a good likelihood of maintaining long-term remission. On the other hand, a calprotectin level of > 300 µg/g in two consecutive samples has been

found to be the best predictor of a flare in patients with ulcerative colitis treated with infliximab[155].

In a study conducted by Ho et al, 90 patients admitted to the hospital for a severe attack of ulcerative colitis were evaluated[156]. In all, 31 patients required a colectomy. In these patients, the calprotectin values at admission were significantly higher than in those not requiring a colectomy. At a cut-off value of 1922 µg/g the sensitivity was low (24.0%), but the specificity was very good (97.4%). A Kaplan-Meier analysis with this cut-off value showed significant differences between the groups.

2 AIMS

The overall aim of this thesis was to evaluate the value of fecal calprotectin to guide treatment and follow-up of patients with IBD, in order to achieve long-term remission. The lack of studies in special clinical situations, the need for standardization of the stool sampling procedure, and a new treatment strategy based on fecal calprotectin monitoring, prompted the following aims:

- To evaluate several aspects of the stool sampling procedure, so as to enable standardization (Paper I).

- To assess whether fecal calprotectin can be used as a surrogate marker of endoscopic disease recurrence 1 year after ileocaecal resection for Crohn's disease (Paper II).

- To test the hypothesis that medical intervention based on regular monitoring of fecal calprotectin can increase the likelihood to maintain remission in patients with ulcerative colitis (Paper III).

- To assess faecal calprotectin as a predictor of disease recurrence in patients with new onset of ulcerative colitis (Paper IV).

3 PATIENTS AND METHODS

Four different patient cohorts were investigated, with one in each of the four papers included in this thesis (Table 1). All the patients were included prospectively. Most of the patients were recruited from the gastroenterology units at Sahlgrenska (Sahlgrenska and Östra) University hospital (Papers II-IV) and South Älvsborgs Hospital, Borås (Papers I-IV). In addition, patients were enrolled at the community hospitals in Skövde (Papers II and III), Varberg (Paper III), Alingsås (Paper III), and Trollhättan (Paper II).

The exclusion criteria common to all the studies were the use of NSAID drugs, pregnancy, and severe comorbidity affecting the ability to comply with the study protocol. The patients' characteristics are presented in detail in the individual papers (I-IV).

All the patients gave written informed consent according to the Declaration of Helsinki. The studies were approved by the Regional Ethical Review Board at the University of Gothenburg.

Table 1. A brief summary of the most important characteristics of the papers.

	Paper I	Paper II	Paper III	Paper IV
No. of patients evaluated	18	30	91	69
Population	UC	CD	UC	UC
Disease characteristics	Active disease	Postoperative follow-up	Monitoring quiescent disease	Newly diagnosed patients
Study objectives	FC distribution, variability and stability. Diary	Endoscopic evaluation. Variability	FC guided treatment	Early prediction of disease course

UC, ulcerative colitis; CD, Crohn's disease; FC, fecal calprotectin

3.1 Paper I

Between January 2012 and May 2013, 18 patients, with a median age of 43 years (range, 18-73), were included. They all had a present flare of ulcerative colitis with mild or moderate disease activity according to the modified Truelove-Witts criteria. The patients had a median Mayo score (Table 3) of 7 (range, 3-9). Eleven patients had left-sided and seven had extensive colitis. Proctitis was an exclusion criterion, as were topical treatments for the colitis and respiratory tract infections.

Two stool samples were collected at every bowel movement on two consecutive days (Figure 1). A diary and a questionnaire concerning the sampling procedure was completed (Appendices A and B). Finally, a flexible sigmoidoscopy was performed to confirm the flare. Ongoing maintenance treatment remained unchanged during the study period, and therapy for the current flare was started after completion of the study.

The distribution of calprotectin in feces, the variability of fecal calprotectin levels during the day and between two consecutive days, and the stability of calprotectin in feces were determined. The influences of stool consistency, fecal blood content, and time between bowel movements were evaluated, as was the stability of the calprotectin in samples stored in room temperature.

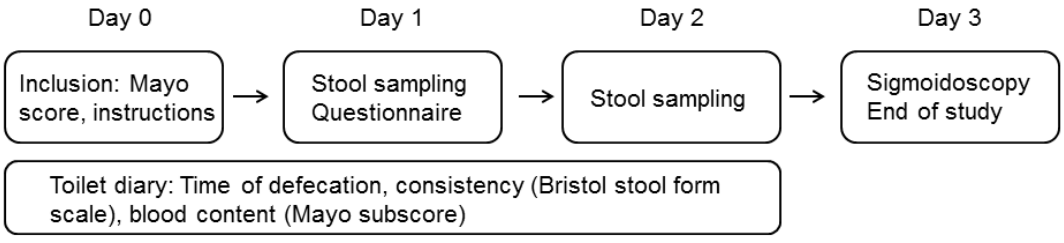

Figure 1. Flow-chart for Paper I.

3.2 Paper II

Adult patients with Crohn's disease confined to the ileocaecal region and who had undergone an ileocaecal resection during the previous year were eligible for this study. In all, 33 patients were included between September 2008 and December 2011. Three patients were excluded, two because of early disease recurrence in the proximal small intestine and one because of withdrawn consent. In 29 of the evaluable patients, the ileocaecal resection performed prior to inclusion in the present study was their first bowel resection. In one patient, a short distal ileal resection that did not include the ileocaecal valve had been performed 5 years earlier. In total, 10 patients were included within 4 months, 4 within 4-8 months, and 16 were included more than 8 months after surgery.

From the time of inclusion to the ileocolonoscopy, a stool sample was sent to the gastroenterology unit in Borås monthly (Figure 2). In a longitudinal part of this study, the variability of the fecal calprotectin levels was determined based on these monthly samples. Every 4 months, blood samples were drawn and disease activity was assessed with the HBI (Table 4).

Twelve months after surgery, an ileocolonoscopy was performed to assess the anastomotic area and the neoterminal ileum according to the Rugeerts' score (Table 2). In a cross-sectional part of the study, the final stool samples, taken at time-points close to the ileocolonoscopy, were used to assess whether the measured levels of calprotectin corresponded to the endoscopic findings.

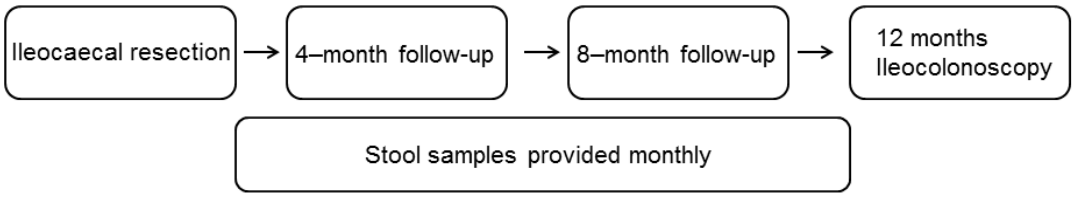

Figure 2. Flow-chart for Paper II.

3.3 Paper III

In all, 109 patients with ulcerative colitis in remission (Mayo score ≤ 2), but with at least one flare during the previous year, were included between August 2009 and December 2012. Eighteen patients were excluded due to protocol violations or failure to deliver a sufficient number of stool samples. Thus, 91 patients were evaluated in the primary outcome analysis. At inclusion, all the patients were on maintenance treatment with an oral 5-ASA agent. The daily dosage did not exceed 2.4 g, 2 g and 4.5 g of Asacol®, Pentasa® or Colazid®, respectively. Patients who were on anti-TNF or corticosteroid therapy were excluded, as were patients with a prior colonic resection.

In this randomized, controlled study, 51 and 40 patients were assigned to the intervention group and the control group, respectively. All patients sent a stool sample via regular mail monthly over a period of 18 months (Figure 3). In the intervention group, a calprotectin value >300 µg/g prompted another stool sample within a week, and if a deviation in the detected concentration of calprotectin above the cut-off level was confirmed, a dose escalation of the 5-ASA agent was accomplished. Accordingly, the dose of Asacol®, Pentasa® or Colazid® was increased to 4.8 g, 4.0 g and 6.75 g, respectively, until the calprotectin level fell to < 200 µg/g or for at least 3 months.

The primary outcome variable was the number of patients who experienced relapse at Month 18. A relapse was defined as an increase of symptoms, consistent with ulcerative colitis, and of sufficient severity to justify a change in treatment.

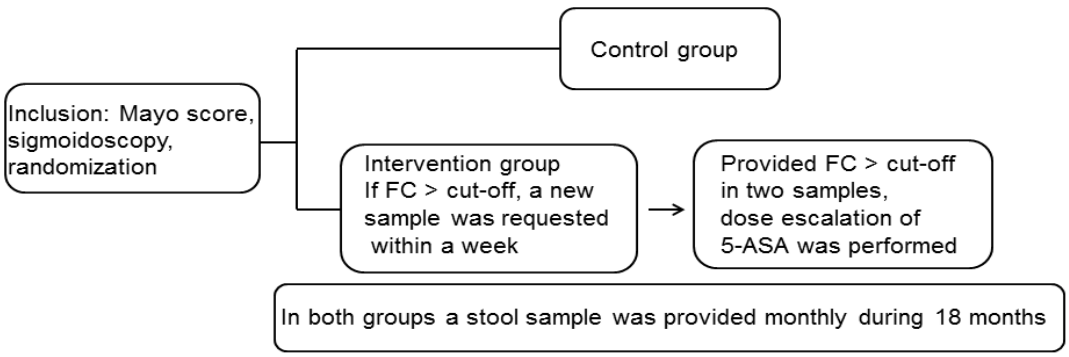

Figure 3. Flow-chart for Paper III.
FC, Fecal calprotectin

3.4 Paper IV

In this study, 69 patients with new onset of ulcerative colitis were evaluated. The median age was 33 (range, 18-74) years, and 13, 18 and 38 patients presented with proctitis, left-sided colitis, and extensive colitis, respectively. Three months after diagnosis, i.e., after an initial individualized therapy based on disease extent and severity, a follow-up was scheduled and the concentrations of calprotectin in the feces were determined.

The values of calprotectin 3 months post-diagnosis were assessed as predictors of the further clinical course (Figure 4). The follow-up period was 3 years. The patients were divided into one group with mild disease course and one group with relapsing disease. The clinical course was considered mild if there was no recurrence during the first year and not more than 1 relapse yearly during the second and third years of follow-up**.** A relapse was defined as an increase in the severity of symptoms, consistent with ulcerative colitis, and of sufficient severity to justify a change in the treatment.

Figure 4. Flow-chart for Paper IV.

3.5 The stool sampling procedure

In all the studies, disposable plastic tubes that included a spoon (Faeces tube; Sarstedt, Nürnbrecht, Germany) were used to obtain stool samples. Typically, 2-3 spoons (approximately 2-3 g) of feces were collected at each sampling time. In Paper I, special feces collection papers (Stuhlfänger; Süsse Labortechnik, Gudensberg, Germany) were dispensed to the participants at the time of inclusion. In Papers II-IV, the patients were informed that it was preferable to collect the stool samples from their first bowel movements in the morning and that sampling should be avoided during menstruation or when suffering from a

respiratory tract infection. The stool samples were delivered by mail (Papers II and III), or directly to the clinic (Papers I and IV). Upon receipt, the samples were frozen at -20°C (Papers II and IV) or -70°C (Papers I and III) prior to analysis.

3.6 Fecal calprotectin analysis

The analyses of the fecal samples were performed by experienced laboratory technicians who had no knowledge of the subjects' clinical status or endoscopic findings. Fecal calprotectin was measured in a quantitative enzyme-linked immunosorbent assay (Calprotectin ELISA; Bühlmann Laboratories AG, Basel, Switzerland) according to the manufacturer's instructions. The intra-assay and inter-assay precisions (coefficients of variation) were declared to be 4.7% and <15%, respectively. According to the manufacturer, the upper limit of normal for faecal calprotectin is 50 μg/g.

Samples were initially diluted 1/50, and if the calprotectin levels were above the standard curve the samples were diluted 1/400. No calprotectin measurements were obtained from extrapolated data. If a noticeable variation between two samples collected from the same bowel movement was recorded, both samples were reanalysed after performing new extractions from the original samples (Paper I).

3.7 Endoscopic evaluation

Colonoscopies and sigmoidoscopies were performed by experienced gastroenterologists who were unaware of the fecal assay results. In all the studies, standard colonoscopes (Olympus CF -H180AL series) were used for both the flexible sigmoidoscopies and the ileocolonoscopies. Bowel preparation prior to ileocolonoscopy was performed according to local routines at the hospitals where the patients were recruited. In most cases 2-4 L of polyethylene glycol (Laxabon®, Movprep®) were prescribed before colonoscopy and a

Microlax® (sodium citrate/sodium lauryl sulfoacetate) enema at a volume of 2 × 5 ml was administered 30-60 minutes before the sigmoidoscopies.

In all the studies biopsies were obtained during endoscopy. Standard preparation of the mucosal specimens and staining with hematoxylin and eosin were performed at the local pathology laboratory.

In patients with ulcerative colitis (Papers I, III, and IV) the endoscopic Mayo subscore (Table 3) was used to assess disease activity[77]. To evaluate endoscopic recurrence in postoperative Crohn´s disease (Paper II), the Rutgeerts' score was used[83]. In Table 2, the 5-graded scoring system is presented in detail. Scores i0 and i1 were considered endoscopic remission and i2 to i4 as endoscopic recurrence.

Table 2. Endoscopic scoring system for postoperative recurrence (Rutgeerts' Score)

	Rutgeerts' score
i_0	No lesions
i_1	≤ 5 aphthous lesions
i_2	> 5 aphthous lesions with normal mucosa between the lesions *or* skip areas of larger lesions *or* lesions confined to the ileocolonic anastomosis
i_3	Diffuse aphthous ileitis with diffusely inflamed mucosa
i_4	Diffuse inflammation with already larger ulcers, nodules, and/or narrowing

3.8 Assessment of clinical disease activity

In Papers I, III, and IV, disease activity was assessed using the Mayo score[77]. This score consists of four items: stool frequency; rectal bleeding; findings of flexible proctosigmoidoscopy; and a physician's global assessment (Table 3). Each item is scored from 0 to 3 and the total score ranges from 0 to 12, with higher scores indicating more severe disease. In our studies, remission was defined as a total score ≤ 2 with no individual subscore >1.

Table 3. The Mayo score, for clinical and endoscopic evaluations of disease activity in patients with ulcerative colitis.

Mayo score
Stool frequency 0=Normal number of stools for this patient. 1=1-2 stools more than normal. 2=3-4 stools more than normal. 3=5 or more stools more than normal.
Rectal bleeding 0=No blood seen. 1=Streaks of blood with stool less than half the time. 2=Obvious blood with stool most of the time. 3=Blood alone passed.
Findings of flexible proctosigmoidoscopy 0=Normal or inactive disease. 1=Mild disease (erythema, decreased vascular pattern, mild friability). 2=Moderate disease (marked erythema, absent vascular pattern, friability, erosions). 3=Severe disease (spontaneous bleeding, ulceration).
Physician's global assessment 0=Normal 1=Mild disease 2=Moderate disease 3=Severe disease

To assess disease activity in Crohn's disease (Paper II), the Harvey Bradshaw Index (HBI) was used[80]. Table 4 shows this index in detail.

Table 4. Harvey Bradshaw index (HBI), used for clinical assessment of disease activity in patients with Crohn's disease.

Harvey Bradshaw index
General well-being 0=Very well, 1=Slightly below par, 2=Poor, 3=Very poor, 4=Terrible
Abdominal pain 0=None, 1=Mild, 2=Moderate, 3=Severe
Number of liquid stools daily
Abdominal mass 0=None, 1=Dubious, 2=Definite, 3=Definite and tender
Complications Arthralgia, uveitis, erythema nodosum, aphthous ulcer, pyoderma gangrenosum, anal fissure, new fistula, abscess (score of 1 per item).

3.9 Diary and questionnaire

A toilet-diary was created for use in the fecal sampling study (Paper I). Details regarding all bowel movements from the time of inclusion to the end of the study had to be recorded, i.e., time of defecation, presence of blood in the stool, and stool consistency (Appendix A). The rectal bleeding subscore in the Mayo score (Table 3) was used to specify the blood content of the stool and the Bristol stool form scale (Appendix C) was used to describe stool consistency. The information given in the diaries was used to perform correlation analyses with the fecal calprotectin levels.

To date, little attention has been paid to patients' opinions concerning the stool sampling procedure[138]. To address this issue, a questionnaire (Appendix B) was constructed (Paper I).

3.10 Statistical Methods

All statistical analyses were performed using the SPSS for Windows software (version 17.0; SPSS Inc., Chicago, IL). Statistical significance was set at $p<0.05$. In Table 5, the essential statistical tests are summarized.

Table 5. Summary of the most important statistical tests used in these studies.

	Paper I	Paper II	Paper III	Paper IV
Pearson's chi-square test		x	x	x
Fisher's exact test		x	x	x
Mann-Whitney U test		x	x	x
Student's *t*-test			x	
Logistic regression				x
Spearman's rho correlation	x			
Intraclass correlation coefficient	x			
Log-rank test			x	x
Coefficient of variation	x	x		

The calprotectin concentrations are presented as the median and inter-quartile range (IQR), whereas other continuous variables mostly were shown as median and range. Categorical variables were presented as percentages.

The fecal calprotectin values were not assumed to be normally distributed. Instead they were skewed, and particularly in Paper II, the sample size was small. Consequently, the nonparametric Mann-Whitney U-test was used to compare the calprotectin levels between groups. Depending on the data distribution, the Student's *t*-test or the Mann-Whitney U-test was used to compare other continuous variables, and the chi-square test was used to compare categorical variables. When the chi-square test was not valid, Fisher's exact test was used.

To evaluate the potential impacts of six different variables on the disease course, logistic regressions were performed (Paper IV). The dependent variable was relapsing disease according to our predetermined definition and the independent variables were: age; sex; smoking habits; extent of disease; CRP; and the Mayo score. Each of these variables was analysed in a model together with the fecal calprotectin concentrations.

In Paper I, Spearman's rho was used to determine the correlation between the calprotectin levels and the data from the toilet-diary. The intraclass correlation coefficient was determined to estimate the correlation between the concentrations of calprotectin in two samples from the same bowel movement. In the same paper the Wilcoxon Signed Rank Test was used to evaluate the stability of calprotectin in stool samples stored at room temperature.

The Kaplan-Meier method was used to derive time-to-relapse curves, and statistical significance was determined using the log-rank test (Papers III and IV).

To determine the variability in fecal calprotectin concentrations during a single day, between two consecutive days, and over time, the coefficient of variation was calculated (Papers I and II).

4 RESULTS

4.1 Issues on the stool sampling procedure (Paper I)

4.1.1 Distribution of calprotectin in feces

In total, 287 samples were delivered from 18 patients with active ulcerative colitis. In 132 pairs of samples, from just as many bowel movements, the correlation (Figure 5) in terms of calprotectin concentrations between the two random samples was determined (ICC = 0.79; 95% CI 0.48 – 0.90).

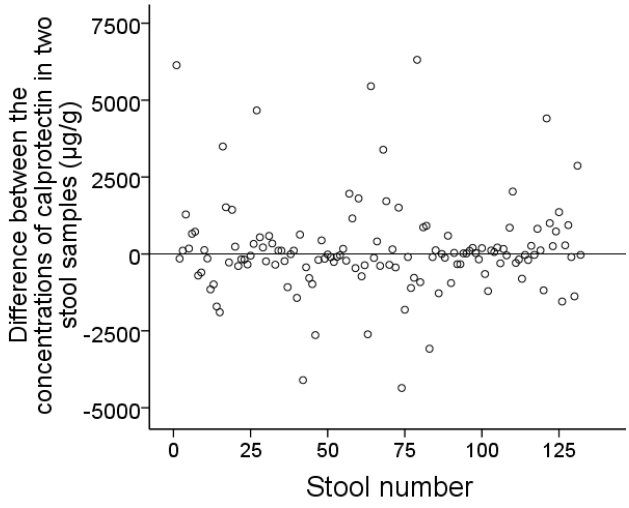

Figure 5. Differences in calprotectin concentrations between two random samples collected from the same bowel movement (n=132) of 17 patients with active ulcerative colitis. The horizontal reference line represents the ideal result when Sample 1 = Sample 2.

4.1.2 Correlations between the calprotectin concentrations and time, consistency and blood content in stool

The calprotectin concentrations correlated significantly with the time between bowel movements (median, r=0.5, range: -0.8 - 0.9; *p*=0.013), as well as the stool consistency (median, r=0.68, range: -0.68 – 0.87; *p*=0.01). As shown in Figure 6, the longer the time between the bowel movements and the looser the

stool consistency, the higher were the concentrations of calprotectin. However, the correlation analysis for the presence of blood in the stool and the level of calprotectin did not reach statistical significance (p=0.057).

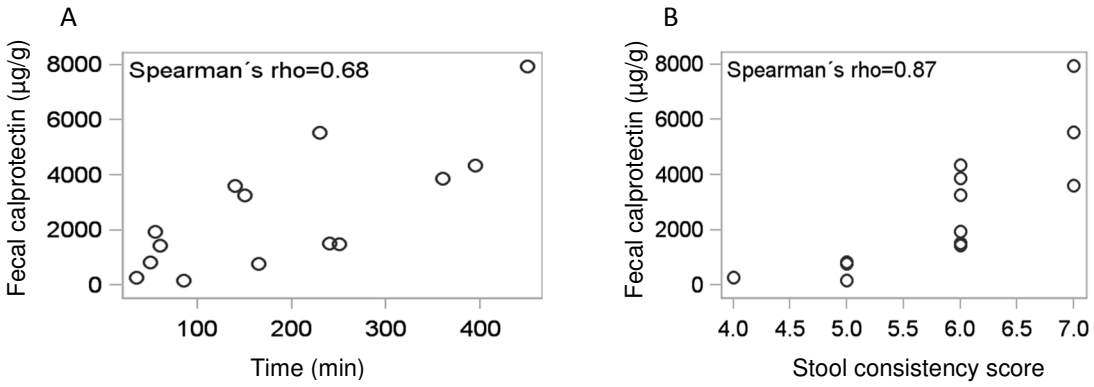

Figure 6. Correlation analyses between fecal calprotectin levels and: (A) time between bowel movements; and (B) stool consistency as assessed with the Bristol stool form scale in one patient with active ulcerative colitis.

4.1.3 Stability of calprotectin

There was no significant difference in calprotectin concentrations between stool samples stored at room temperature for 1 day and for 3 days. However, after 7 days at room temperature, the calprotectin levels showed a mean decrease of 28% (p<0.01; 95% CI 0.10-0.47).

4.1.4 Questionnaire

Overall, 17 patients answered the questionnaire. It was obvious that the stool sampling procedure was not burdensome for the patients. A large majority of the patients found it *acceptable* or *without problems* to handle the collection devices.

Comments. We found a very good correlation between the calprotectin values in two random samples obtained from the same bowel movement. This is consistent with the results reported in previous studies. Moreover, to avoid misleadingly low values, stool samples should preferably be collected from the first bowel movement in the morning.

4.2 Fecal calprotectin to assess endoscopic recurrence in postoperative Crohn's disease (Paper II)

In 30 patients, ileocolonoscopies were performed after a median time of 369 (287-434) days after the ileocaecal resection. The endoscopic scores were i0 and i1 in 6 and 11 patients, respectively, and i2 and i3 in 10 and 3 patients respectively. No patient was scored as i4. There was no significant difference in the calprotectin levels between those with endoscopic disease recurrence (i2-i3) and those without [median (IQR): 227 (120-1066) µg/g vs 189 (75-364) µg/g; *p* = 0.25]. Nevertheless, there was a trend, since 6 (75%) of 8 patients with calprotectin levels < 100 µg/g were in endoscopic remission, whereas 6 (86%) of 7 patients with high levels of calprotectin (> 600 µg/g) had an endoscopic recurrence (Figure 7).

We found no significant differences in the laboratory parameters or the HBI scores between the two groups of patients.

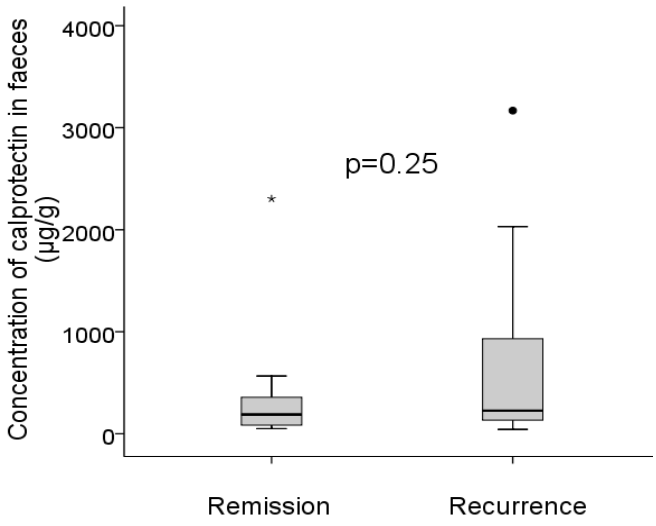

Figure 7. Concentrations of fecal calprotectin in 30 patients one year after ileocaecal resection for Crohn's disease. Endoscopic remission (n=17) is defined as a Rutgeerts' score i0-i1, and recurrence (n=13) as score i2-i4.

Comments. It could be argued that only a few patients had severe endoscopic disease recurrence, since only three patients were scored as i3 and no patient was scored as i4.

4.3 The variability of fecal calprotectin (Papers I and II)

The variability of the concentrations of calprotectin was evaluated during a single day and between two consecutive days in patients with active ulcerative colitis, as well as in monthly samples from patients with clinically inactive postoperative Crohn's disease.

In patients with active ulcerative colitis, the variability of the fecal calprotectin levels during a single day was high for most of the patients (Figure 8). The median coefficient of variation (CV) was 52% (range, 4-178). The variation was most pronounced in patients who had high levels of calprotectin. On the other hand, in six patients with active ulcerative colitis, one patient had a calprotectin value < 50µg/g, whereas in another two patients the value fluctuated below 100 µg/g, and in additionally three patients values < 250 µg/g were noted. In other samples obtained during the same day, all these six patients had high levels (> 800 µg/g) of calprotectin.

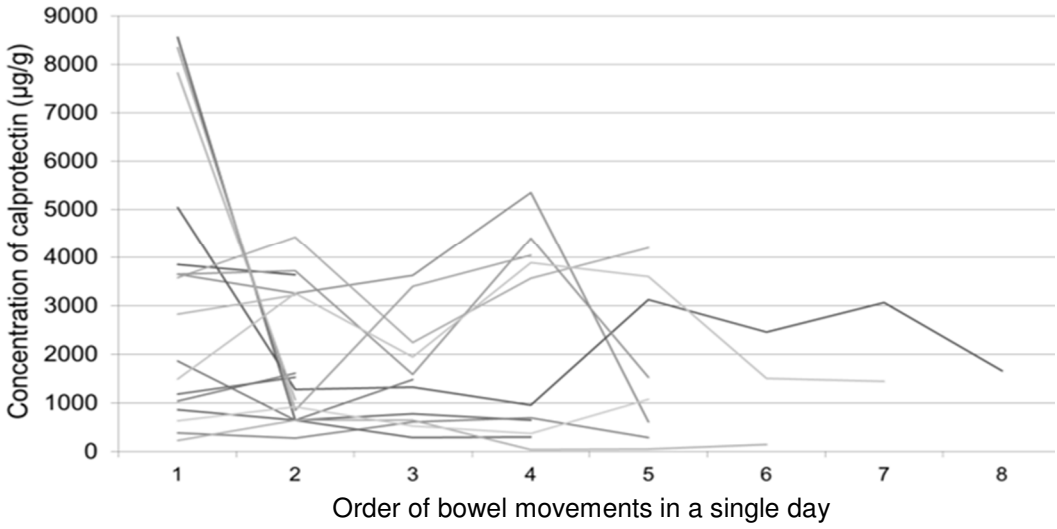

Figure 8. The figure shows the individual concentrations of calprotectin in feces, obtained from 17 patients with active ulcerative colitis during a single day. The values on the x-axis represent the bowel movements in sequence, i.e., 1 is the first bowel movement during the day, 2 is the second bowel movement, and so on.

The day-to-day variability was determined using the calprotectin values from the first defecations in the morning. This calculation gave a somewhat lower CV, with a median of 40.8% (range, 3.1-127.8).

In Figure 9, the variations in fecal calprotectin levels among the monthly samples from patients with a newly performed ileocaecal resection are shown. The median CV was 63.0% (range, 21.5-99.4). The median values for all calprotectin values at each month were 62-205 µg/g.

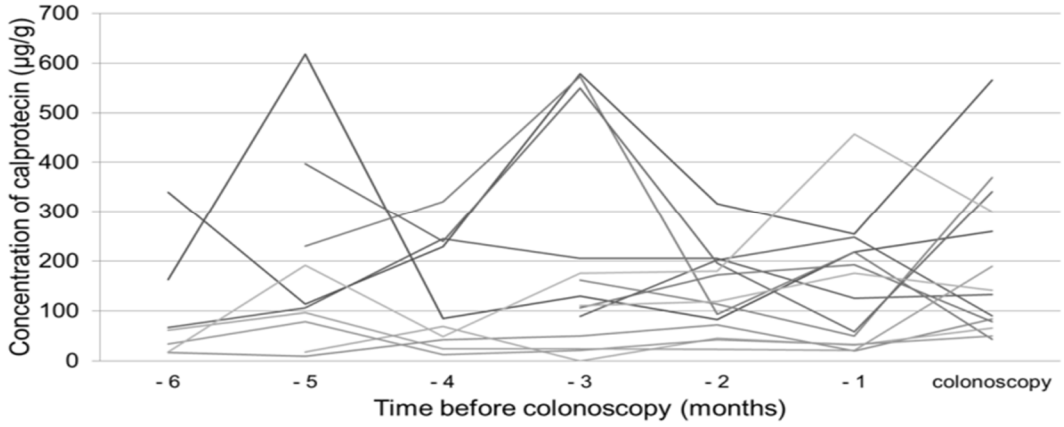

Figure 9. Concentrations of fecal calprotectin in fecal samples obtained monthly from 14 patients with Crohn´s disease. All the patients had an ileocaecal resection performed 1 year prior to the colonoscopy.

4.4 Fecal calprotectin to guide treatment in ulcerative colitis (Paper III)

The patients in the intervention group (n=51) and the control group (n=40) delivered 800 (mean, 15.7/patient) and 554 (mean,13.8/patient) stool samples, respectively. There was no statistically significant difference (p=0.91) between the levels of calprotectin in all these samples [median (IQR): 82 (34-310) µg/g vs 86 (37-278) µg/g]. Most of the patients (87.9%) were on treatment with Asacol (1.6-2.4 g) at inclusion. A fecal calprotectin concentration of 300 µg/g was set as the cut-off for intervention. As shown in Figure 10, intervention, i.e., dose escalation of the 5-ASA agent, was accomplished in 28 patients, of whom 8 (28.6%) suffered a relapse. In the control group, significantly more patients

(57.1%) with a calprotectin level >300 µg/g experienced a relapse. However, there was no significant difference in the relapse rates overall between the patients in the intervention group and the control group (Figure 11). In 10 (55.6%) of the 18 patients who experienced a relapse in the intervention group, the calprotectin value did not reach the cut-off value before they relapsed.

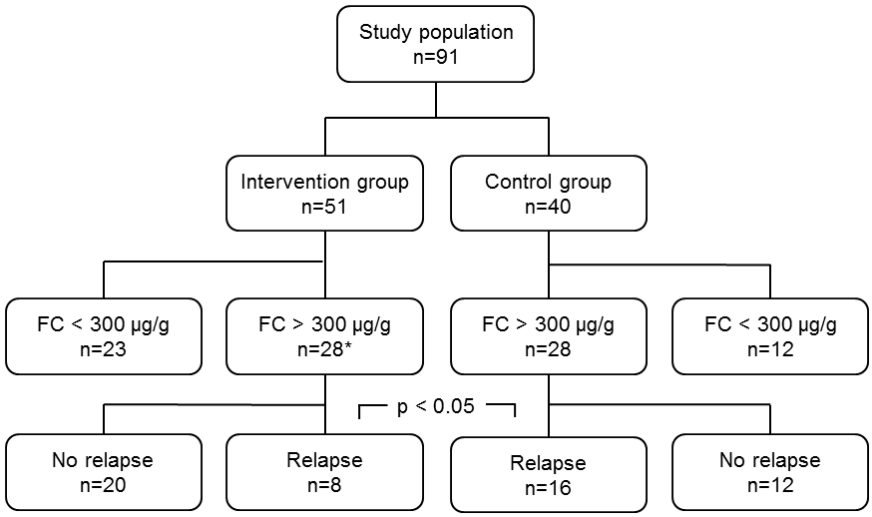

Figure 10. Disposition and outcomes for patients in the primary efficacy population.

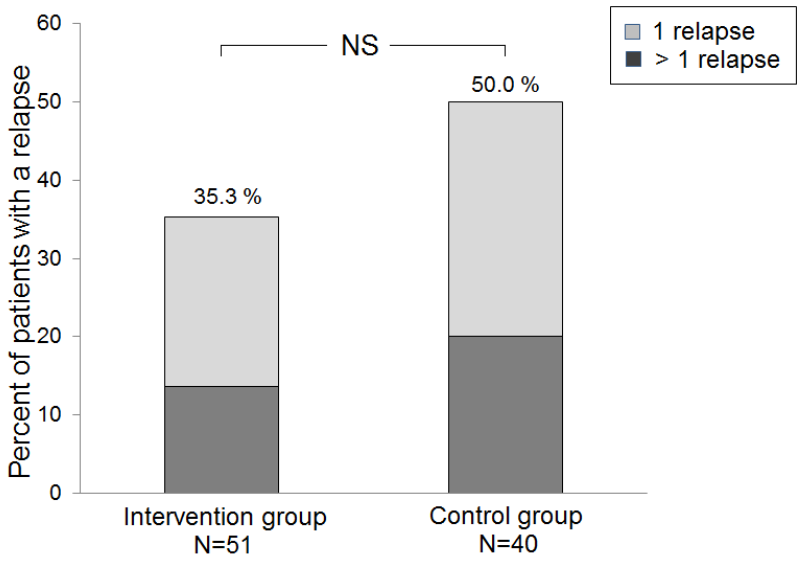

Figure 11. Proportion of patients with ulcerative colitis and at least one disease relapse. The patients in the intervention group performed a dose escalation of ongoing 5-ASA treatment if the fecal calprotectin level exceeded 300 µg/g.

As shown in Figure 12, the survival curves for the two groups are clearly separate, although statistical significance is not achieved.

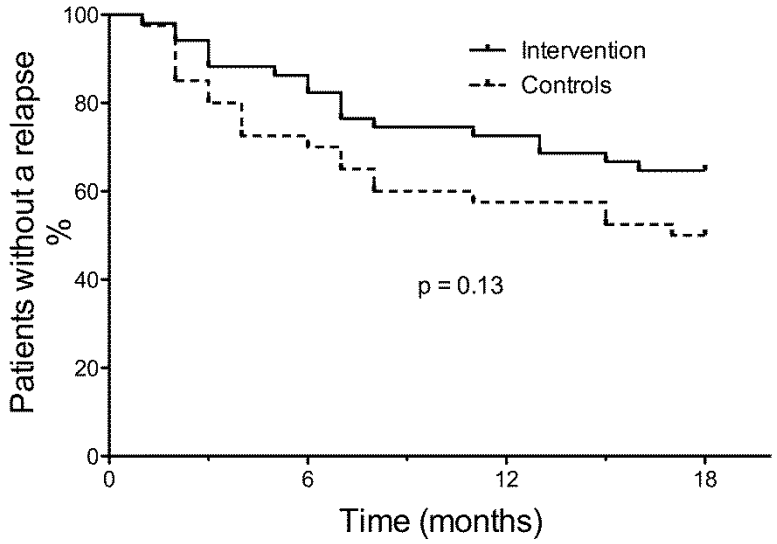

Figure 12. Kaplan-Meier time-to-first relapse curves for patients with ulcerative colitis in the active intervention and the control groups. Intervention involved a dose escalation of a 5-ASA agent when the fecal calprotectin levels in the monthly delivered stool samples exceeded the predetermined cut-off level of 300 µg/g.

Comments. In the analysis of the overall relapse rates, as well as the survival analysis a trend towards differences between the groups was seen. However, statistical significance was not achieved. One possible explanation for this might be a type II error.

4.5 Fecal calprotectin to predict the clinical course in patients with newly diagnosed ulcerative colitis (Paper IV)

The 1-year follow-up included 69 patients. Thereafter, 2 patients were lost to follow-up, resulting in 67 patients to evaluate at the 2-year and 3-year follow-ups. The concentrations of fecal calprotectin 3 months after the diagnosis of ulcerative colitis was established were compared for patients with a mild disease course and those with relapsing disease (defined in the *Patients and Methods*

section) at 1, 2 and 3 years. Moreover, the fecal calprotectin values at 3 months were assessed as markers to predict relapse.

Three months after diagnosis, 60 (87.0%) patients were in remission (Mayo score ≤ 2). After 1 year, 24 and 45 patients had experienced a mild (i.e.,no relapse) and a relapsing (i.e., ≥1 relapse) disease course, respectively. As shown in Figure 13A, the concentrations of fecal calprotectin at the 3-month follow-up were significantly higher in the patients with a relapsing disease course during the first year than in those with a mild disease course [median (IQR): 263 (100-634) µg/g vs 102 (38-225) µg/g; *p*=0.009].

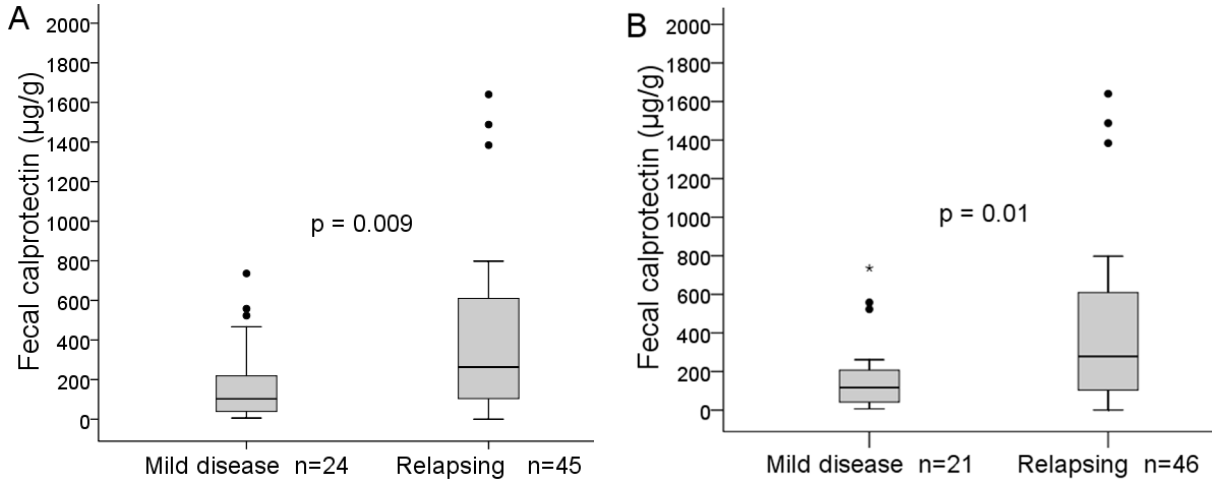

Figure 13. Fecal calprotectin levels 3 months after initial therapy for new onset of ulcerative colitis in 69 and 67 patients, respectively. The patients are distributed into two groups based on the clinical course during 1 year (A) and 3 years (B). Mild disease is defined as no recurrence during the first year and not more than one relapse yearly during the second and third years of follow-up.

After 3 years of follow-up (Figure 13B), the difference in the fecal calprotectin levels, in samples obtained at the 3-month follow-up, was still significant between the groups [median (IQR): 280 (112-622) µg/g vs 118 (39-219) µg/g; *p*=0.01].

To assess the global yield of calprotectin to predict the clinical course and determine the optimal cut-off value, receiver operating characteristic curves were constructed to calculate the area under the curve (AUC). The AUC values

at 1 year and 3 years were 0.69 ($p < 0.01$) and 0.70 (p=0.01), respectively. The highest sum value of the sensitivity and specificity was found for fecal calprotectin values of 169 µg/g and 262 µg/g after 1 year and 3 years follow-up, respectively (Table 6).

Table 6. Sensitivity, specificity, and the positive and negative predictive values for calprotectin to predict the clinical course.

	1 year [a]	3 years [b]
Sensitivity, %	64.4	52.2
Specificity, %	70.8	85.7
Positive predictive value, %	80.6	88.9
Negative predictive value, %	51.5	45.9

[a] Fecal calprotectin cut-off of 169 µg/g
[b] Fecal calprotectin cut-off of 262 µg/g

Figure 14 shows time-to-relapse curves according to the Kaplan-Meier method using a fecal calprotectin level greater than or less than 262 µg/g.

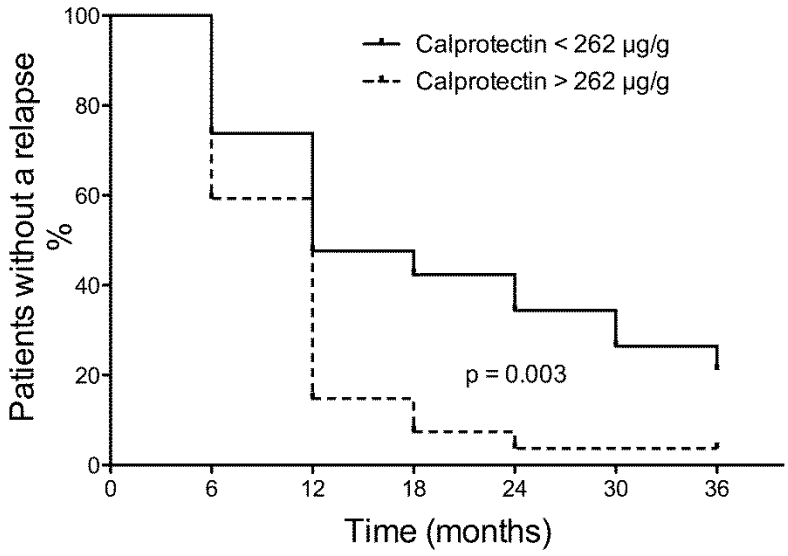

Figure 14. Kaplan-Meier time-to-relapse curves for patients with ulcerative colitis in relation to fecal calprotectin levels at the 3-month follow-up (either >262 µg/g or <262 µg/g).

The logistic regression analysis revealed that the concentrations of calprotectin at the 3-month follow-up (at 1 year: p=0.007, odds ratio [OR]=4.0; at 3 years:

p=0.009, OR=4.31) and age (at 1 year: *p*=0.019, OR=0.95; at 3 years: *p*=0.003, OR=0.93) were the only variables that significantly predicted the clinical course during 1 year and 3 years.

Comments. The administered therapy is described in detail in the full paper (Paper IV). A predetermined treatment strategy was not included in the protocol. However, to provide as uniform treatment as possible, a limited number of physicians was responsible for the follow-up, and therapy was given in accordance with current practice.

Altogether, 13 patients had disease limited to the rectum. It can be argued that interpretation of fecal calprotectin levels in these patients might be problematic. Therefore, all the analyses were also performed while excluding these patients, and similar results were obtained.

5 DISCUSSION

5.1 Summary

A summary of the usefulness of determining the fecal concentrations of calprotectin in patients with IBD could be very brief. The level of calprotectin correlates with the level of inflammatory activity, and is the best available marker of mucosal inflammation. High levels of calprotectin indicate a need for further investigation to establish a diagnosis, or in patients with known IBD, to consider a change in therapy with or without further investigation. Finally, increasing levels of calprotectin in asymptomatic patients reflect subclinical inflammation, and accordingly, an increased risk of disease recurrence. This is consistent with previous reports that normal levels of fecal calprotectin predict mucosal healing with high probability, and that mucosal healing has a strong impact on the clinical course of IBD.

The present thesis deals with the clinical utility of fecal calprotectin in specific situations. Our results are in the main consistent with the principal features of calprotectin and reveal some new and interesting data.

To date, little attention has been focused on the importance of the stool collection procedure. Based on the results in Paper I, we recommend sampling from the first bowel movement in the morning, since the longer the time between bowel movements, the higher are the levels of calprotectin. This minimizes the risk of misleadingly low values for fecal calprotectin.

According to the present results, fecal calprotectin do not replace an ileocolonoscopy in the postoperative setting in patients with Crohn´s disease (Paper II). However, a large majority of the patients with calprotectin values below 100 µg/g were found to be in endoscopic remission, and in those a colonoscopy may be avoided.

A substantial and clinically important intra-individual variability of fecal calprotectin has been confirmed (Papers I and II). Further research is needed to find additional reasons for this and to develop methods to reduce the impact of the variability as much as possible.

To reduce the risk of a clinical relapse, therapeutic efforts, based on regular monitoring of fecal calprotectin levels in asymptomatic patients with ulcerative colitis, were implemented in Paper III. The results raise the possibility of a new treatment strategy, to identify and treat patients with quiescent ulcerative colitis at risk of impending flare before symptoms appear.

In Paper IV, we demonstrate that the levels of fecal calprotectin after initial treatment, are predictive of the further clinical course in patients with new onset of ulcerative colitis.

5.2 Issues on the stool sampling procedure

To obtain a fecal specimen for biochemical analysis is not as simple as to draw a blood sample. Several sources of errors may arise, such as an uneven distribution of the agent of interest, contamination with water or detergents from the toilet basin, and the presence of fibers and other solid particles in the stool. Although used for several years in clinical practice, an accepted standardized protocol for collecting stool samples for the analysis of biomarkers, such as calprotectin, has not yet been established. Actually, few studies have been designed to assess the extents to which different aspects of the sampling procedure influence the results when analyzing fecal calprotectin. In early studies, stools were collected over 24 hours or even over several consecutive days to analyze it for calprotectin. Thereafter, all studies have been performed using small randomly collected stool samples, typically containing a few grams of feces. Thus, an even distribution of calprotectin in feces is crucial to accept such a practice. This has been satisfactorily confirmed in studies that have compared the concentrations of calprotectin in randomly obtained small samples with the concentrations in 24-hour homogenized specimens or that have compared multiple small samples from the same stool specimen[125, 132, 157-161]. Other fecal biomarkers have been assessed in a similar way, with the same outcome[162, 163]. Since the fecal stream is in direct contact with the intestinal mucosa, the incorporation of molecules released from inflamed or damaged mucosa into the fecal stream is possible. Apparently, the fecal compounds are mixed adequately during the passage through the gastrointestinal tract, probably due to peristaltic propulsions.

The present results are in line with those of previous reports. After careful instructions to obtain two separate samples from opposite ends of each stool, caught in the feces collection paper, pairs of samples obtained by the patients themselves were compared, whereas in most previous studies, the samples were prepared in laboratory. For those stool specimens that showed large differences between the two samples, we reanalyzed the samples after new extractions to confirm the results. An ICC of 0.79 indicates strong agreement between the two samples. In previous studies, the coefficient of variation, if several samples were compared[157, 159], and Pearson's or Spearman's correlation coefficient, if pairs of samples were compared[125, 132], were calculated. Using Pearson's and Spearman's correlation tests in the present situation might involve a risk to overestimate the level of agreement if there is a systematic difference between the two datasets.

Published data on the stability of calprotectin in feces are scarce[125, 157, 158]. Although it is widely accepted that calprotectin remains stable for 3-7 days in stool samples stored at room temperature, we decided to repeat this assessment. We found that the concentrations of calprotectin were unchanged after 3 days at room temperature, whereas there was a significant decrease after 7 days. Patients with active ulcerative colitis were included in this analysis: in 2 of the 18 included patients, the calprotectin levels decreased from > 250 µg/g on Day 1 to < 250 µg/g on Day 7. Thus, the clinical importance of the observed decrease over a week is negligible for most patients. However, this issue may be of importance if the calprotectin levels are close to the determined cut-off. Although, storage at room temperature for more than 3 days cannot be recommended, this does not impair the possibility to send samples via regular mail.

Some concerns about whether patients would be willing to collect fecal specimens have been expressed and evaluated in other situations. In a screening study for colorectal cancer using fecal occult blood testing, patients expressed the unpleasantness to collect stool samples as a major reason for refusing participation, and in another study evaluating a *Helicobacter pylori* stool antigen test, stool handling was identified as an obstacle to patient compliance[164, 165]. In patients with a chronic bowel disorder, such as IBD, the opinions might be

different. In a questionnaire (Appendix B), we asked patients with active ulcerative colitis about several aspects concerning stool sampling. The patients expressed no reluctance to collect the stool samples, and they declared that it was not burdensome. In a study by Schoepfer et al, the feasibility of collecting fecal specimens for calprotectin analysis was examined in 38 outpatients who were provided with a special stool sampling kit[138]. The compliance was excellent: 95% of the patients found the collection to be straightforward after instructions and would do it again.

It is evident from our study that most patients preferred to use the collection paper instead of no collection paper at all at the next sampling occasion. At inclusion, sampling sets, including feces collection papers, were provided, and we concluded that adequate equipment should be recommended to facilitate the collection procedure for the patients and to avoid artefacts associated with sampling from the toilet water. Hitherto, a spoon connected to a screw cap has been commonly used, whereby 1-3 spoons of feces are placed in a plastic tube. To make sampling even easier, commercial sets with new devices for stool collection have been introduced. One example is a rod with radial grooves[166]. This rod must be dipped into the stools to fill the grooves with feces and is thereafter placed in a tube that contains the extraction solution. This procedure allows sampling of the volume of feces required without weighing the sample. Thus, the procedure is simplified for the patients and the laboratory personnel.

During two consecutive days, the patients entered into a diary various information about the time of defecation, the presence of blood, and stool consistency. This information proved to be very useful. Interestingly, we found that the longer the time interval between bowel movements, the higher became the concentrations of calprotectin. In some papers, the advantage of collecting stool from the first bowel movement has been expressed. This is based on the reasonable assumption that leukocyte-derived proteins released into the gut lumen from the inflamed mucosa accumulate in the lumen between the bowel movements and are drained during defecation. Consequently, the fecal concentrations of these leukocytic proteins are highest in samples collected when there is long interval between the defecations. This hypothesis is supported by our results. A Danish group compared the calprotectin levels in the morning with the levels in the afternoon, and found no difference for patients with IBD[167]. However, in that study, the mean value from samples obtained

during the morning was compared with the corresponding mean value during the afternoon.

Our results are of great clinical importance, in particular as we could demonstrate that some patients exhibit large variations in their calprotectin levels from samples collected during the same day. To avoid false low values of calprotectin, we suggest that stool samples should preferably be obtained from the first defecation in the morning, provided that the patient did not pass stool during the night. Furthermore, to be able to compare the values over time, the patients should be recommended to always take their samples with approximately the same time interval since the previous bowel movement.

We also found a significant correlation between stool consistency and the level of calprotectin in feces. Higher values of calprotectin in loose stools are probably a consequence of the disease activity in the present study. The impact of stool consistency on the calprotectin values has been reported previously, although the clinical significance is unclear but probably low[160, 168]. We also found a trend towards a correlation between the self-estimated content of blood in the stool and the level of calprotectin. We are inclined to think that this is also a consequence of disease activity, since considerable amounts of blood would be required to increase significantly the calprotectin levels[161, 169]. On the other hand, we and others suspect that pus and mucus in the stool can affect the calprotectin values for patients with active ulcerative colitis, and this may to some extent explain the variability seen in these patients[167].

To summarize, the details of the stool sampling procedure are of importance for the results obtained when analyzing fecal specimens for the content of calprotectin. We show that: 1) the distribution of calprotectin in stool specimens does not affect negatively the utility of small randomly obtained samples; 2) the longer the time interval between the bowel movements, the higher are the levels of calprotectin in patients with active ulcerative colitis; 3) stool sampling with designed kits is not burdensome for the patients; and 4) calprotectin is stable in stool samples stored at room temperature for 3 days. Accordingly, we suggest that stool samples should be obtained using adequate equipment, at the first bowel movement in the morning, and if appropriate, that stool samples should be sent by post during weekdays, avoiding delayed delivery over weekends. Further work is needed to ensure the quality of stool sampling and to standardize

the procedure. Until that time, numerous instructions are available via the internet and for those who are interested, instructive, and rather amusing videos can be found on YouTube.

5.3 Fecal calprotectin to assess endoscopic recurrence in postoperative Crohn´s disease

Rutgeerts and coauthors have demonstrated that endoscopic recurrence precedes clinical recurrence in patients with Crohn's disease, and that the severity of the endoscopic recurrence, assessed within the first year after ileocaecal resection, is predictive of the subsequent clinical course[83, 170]. They studied 89 patients, of whom 90% underwent first resections, 92% underwent ileocecostomy or ileotransversostomy, and a few a more distal ileocolonic anastomosis. No medical treatment was given after resection. The severity of the endoscopic recurrence, in terms of ulcers and diffuse inflammation in the neoterminal ileum, was assessed according to a 5-graded scoring system (Table 2). More than 70% of the patients had any endoscopic recurrence, whereas only 20% presented symptoms one year after the resection. Symptomatic relapses in subsequent years were strongly associated with the severity of the endoscopic lesions at the 1-year ileocolonoscopy. Patients with no or only mild lesions, i.e., i0 and i1, generally maintained long-term clinical remission, whereas patients with diffuse ileitis as well as large ulcers, i.e., i3-i4 had a poor prognosis. Commonly, i0 and i1 are considered endoscopic remission and i2 to i4 an endoscopic recurrence. Referring to these data, an endoscopic evaluation of the neoterminal ileum and the anastomosis within 1 year after surgery are recommended for prognostic purposes and to guide further treatment[61].

The concentrations of fecal calprotectin correlate significantly with the endoscopic disease activity in Crohn's disease[91, 124, 171-174]. The role of fecal calprotectin in correctly identifying patients with an endoscopic disease recurrence in the postoperative setting has been evaluated in some studies with various outcomes[175-180]. We decided to perform a study with a homogeneous group of patients assessed according to the original scoring system[83], to determine the clinical utility of fecal calprotectin in patients with Crohn's disease 1 year after ileocaecal resection. In the present study, the fecal calprotectin levels did not discriminate between patients with endoscopic

disease recurrence and those in endoscopic remission. We also considered the previously suggested cut-off values for endoscopically active disease, i.e., 200 and 250 µg/g, but the results were discouraging. Therefore, we conclude that fecal calprotectin levels do not replace an ileocolonoscopy in the postoperative setting in patients with Crohn's disease. However, a large majority of the patients with calprotectin levels <100 µg/g were in endoscopic remission, and in those a colonoscopy may be avoided. At this cut-off, the sensitivity was 85% for calprotectin to identify correctly patients with endoscopic recurrence, but the specificity was poor.

Previously published studies on this topic are small and the reported results are not consistent. In one study, there was a long time between the stool sampling and colonoscopy, while in two other studies there were no significant differences in the fecal calprotectin levels between patients with endoscopic recurrence and those in remission [175, 177, 178]. In two of these studies, definitions other than Rutgeerts' score were used for the endoscopic examination[177, 178]. In contrast, a good correlation between the endoscopic findings and the levels of calprotectin was reported in other papers[176, 180, 181]. In these three latter studies, endoscopic recurrence was defined as a Rutgeerts' score ≥i2. In preliminary data from a large cohort (the POCER study), the fecal calprotectin levels correlated with endoscopic scores and a value >100 µg/g was suggestive of endoscopic recurrence, i.e., an indication for colonoscopy[182].

Several differences can be identified between all these studies, including ours, and to some extent they explain the different results: the ileocolonoscopies were performed at different time intervals after the surgical resections; different types of resections were performed (ileal, ileocolonic and colonic); the Rutgeerts' score was used in different ways (anastomotic lesions included or not); and the endoscopic findings were assessed during or after the endoscopy. In most of these studies, endoscopic assessment of the colon was not mentioned, (but we must believe that it was normal), the histology was not reported, and most importantly, an examination of the complete small bowel was not performed. Moreover, and maybe most noteworthy, all the studies are small, including only 12-30 patients, and it is noticeable that the proportion of patients with severe endoscopic recurrence (i3-i4) is very small in all the studies. Accordingly, many of the patients with a recurrence belong to the intermediate risk group (i2).

Our present study, which is the largest published study to date that uses Rutgeerts' score to evaluate fecal calprotectin in this setting, has important strengths. All 30 patients were evaluated close to 1 year after surgery, and they had similar resections and an ileocolonic anastomosis. Furthermore, the original definition of the scoring system was used. However, criticism has been directed against Rutgeerts's scoring system[74, 183], in particular the difficulty associated with correctly assessing anastomotic lesions (i2). Furthermore, it is a subjective assessment and even if the definitions in the score appear to be distinct, there is room for individual interpretation. The difference between endoscopic remission (i1) and endoscopic recurrence (i2) is just one aphthous ulcer, which can be easily missed during endoscopy. Thus, the i2 score could be the uncertain score upon which much depend in the studies mentioned. Furthermore, the impact of one additional aphthous ulcer on the level of fecal calprotectin is uncertain.

Examination of the complete small bowel was not performed in the studies referred to above. This illustrates the complexity of evaluating disease activity in Crohn's disease, as compared to ulcerative colitis. The performance of fecal calprotectin as a biomarker of disease activity in Crohn's disease has mainly been assessed using ileocolonoscopy. In recent studies (Table 7), which present the results from 87-210 ileocolonoscopies performed in patients with Crohn's disease and various disease activity levels, significant correlations between the endoscopic disease activity scores and the fecal calprotectin concentrations have been reported[91, 124, 171-174, 180]. Moreover, none of the serum biomarkers or clinical indices performed better than fecal calprotectin.

Table 7. Correlation of fecal calprotectin levels with endoscopic activity in patients with Crohn's disease, and suggested optimal cut-off values for endoscopic remission.

Author	No. of endoscopies	Endoscopic Activity Index	Correlation with fecal calprotectin	Calprotectin cut-off in inactive CD (µg/g)
Sipponen [91]	106	CDEIS	0.73	200
Jones[172]	164	SES-CD	0.45	--
Sipponen [171]	87	SES-CD	0.64	166
Schoepfer [173]	140	SES-CD	0.75	70
D´Haens [124]	87	CDEIS	0.42	250
af Björkesten [174]	210	SES-CD	0.56	94
Lobatón [180]	115	CDEIS	0.72	274

CD, Crohn's disease; CDEIS, Crohn's disease Endoscopic Index of Severity; SES-CD, Simple Endoscopic Score for Crohn's Disease.

The highest values for calprotectin and the best correlations between endoscopy and calprotectin have been documented for patients with colonic or ileocolonic disease. In patients with isolated ileal disease, no or at best poor correlations between endoscopy and calprotectin were reported in some studies[91, 171, 172, 180]. For active disease, D´Haens et al suggested a cut-off value for fecal calprotectin of 250 µg/g. The sensitivity and specificity for the presence of any ulcers were 51.6% and 82.6%, respectively. The performance was improved if patients with purely ileal disease were excluded[124]. In Finland, in particular by Taina Sipponen and co-workers, great efforts have been made to evaluate the utility of fecal calprotectin in Crohn's disease. An ileocolonoscopy for the follow-up of anti-TNF therapy was performed in 210 patients, and calprotectin value of 94 µg/g was found to be the best cut-off for endoscopic remission (SES-CD 0-2), with a sensitivity and specificity of 84% and 74%, respectively. A similar result with the same cut-off was reported for patients with complete mucosal healing. In contrast to most studies, this group reports endoscopically active disease in 13% of the patients who have a normal level of calprotectin (< 100 µg/g). In other studies, a normal level of calprotectin has indicated mucosal healing with good precision, in both Crohn's disease and in ulcerative colitis[171, 184-186].

The use of fecal calprotectin as a biomarker of disease activity in the small intestine has been evaluated in a few studies using capsule endoscopy. In a study by Jensen et al, the performance was equally good in patients with colonic, ileocolonic and isolated small bowel Crohn's disease[187]. In another study, fecal calprotectin was found to be a good predictor of small intestine findings for the selection of patients to undergo small bowel capsule endoscopy after negative colonoscopy and gastroscopy [188]. In contrast, Sipponen et al showed poor precision for calprotectin and S100A12 as biomarkers in a similar study[189].

In summary, the levels of fecal calprotectin offer the best precision for the assessment of disease activity in patients with Crohn's disease as compared with serologic biomarkers and clinical activity indices. However, and importantly, it seems that the levels of calprotectin in patients with ileal disease are lower than in those with colonic and ileocolonic disease. Furthermore, in some studies, no or only poor correlations between fecal calprotectin and endoscopic disease

activity in the ileum were reported. Thus, it is not surprising that the results are inconsistent in the postoperative setting. Nonetheless, most of the studies, including ours, report a high probability of endoscopic remission when fecal calprotectin is normal or only slightly elevated. This is a central outcome because it suggests that postoperatively, a colonoscopy might be avoided. Moreover, patients with Crohn's disease need accurate follow-up and monitoring of disease activity on a regular basis. Consequently, in clinical practice, decisions regarding therapy should not be based on a single laboratory marker, but rather on regular monitoring and repeated tests followed by adequate investigation if the test values increase. Long-time follow-up of our 30 patients would be of interest to determine if the endoscopic assessment or fecal calprotectin levels best predict the clinical course.

5.4 The variability of fecal calprotectin

The variability of calprotectin within a single stool sample is very low, as discussed in a previous section, but what about the variability between stools? First, the variability of fecal calprotectin is usually determined using the coefficient of variation (CV). This coefficient is a normalized standard deviation expressing the standard deviation (SD) as a proportion of the mean ($\bar{x}$) value, CV= $SD/\bar{x} \cdot 100$. The higher the CV, the greater is the dispersion of the data. The CV is commonly used to assess the reliability of laboratory techniques. The advantage of CV is that the variabilities in series of values of various magnitudes or various units can be compared. Consequently, the CVs in the following three series of values are identical: 1, 2, 3, 4, 5 and 100, 200, 300, 400, 500 and 1000, 2000, 3000, 4000, 5000 (CV= 52.7%). This level of dispersion is quite high.

Coming back to calprotectin, let us pretend that those three numeric series are values (µg/g) of fecal calprotectin. In a clinical perspective, the variation in the second series (100-500 µg/g) is the only one of importance, although all the series have the same variation expressed in terms of CV. This example demonstrates the difficulty associated with interpreting the CV and the importance of having an idea of the magnitude of the calprotectin results before a conclusion is stipulated. To present the results using other descriptive methods

and/or to present the results in a clinical content is necessary, and it might better describe the variation of the calprotectin values than the CV alone.

Even during a single day we found high CV values for patients with active ulcerative colitis. Similar results were reported by the Danish group[167]. The variation was most pronounced in patients with high levels of calprotectin and overall, the variability increased with higher levels of calprotectin. Variation of the calprotectin levels throughout the day is of no clinical relevance for these patients. However, in one-third of the study population there was a fluctuation below 250 µg/g in at least one sample during the day. This level of fecal calprotectin has been recommended as the most relevant cut-off value to distinguish endoscopically active disease from inactive disease[124, 190]. All these patients undoubtedly had increased calprotectin values in other samples during the same day. Furthermore, in contrast to studies that have compared groups of patients[126, 191-193], it is not possible to estimate the disease severity in an individual symptomatic patient based on a single calprotectin value.

Variability of calprotectin in stool specimens collected on separate days has been demonstrated in patients with IBD, colorectal cancer, as well as in healthy controls[127, 132, 159, 161, 194, 195]. In 14 patients (Paper I) with active ulcerative colitis, the median intraindividual CV for the calprotectin levels in stool samples collected at the first bowel movements in the morning on two consecutive days was 40.8% (range, 3.1-127.8). Thus, lower variability was found for the samples from the morning than for the samples collected during the same day. A possible explanation for this is that the calprotectin values in the morning better reflect the disease activity, which in all likelihood is very much the same on Day 1 and Day 2. Another simpler explanation could be that the calculations of CV during separate days were based on more (2-8) values than the day-to-day variability. In few patients, the calprotectin levels in any of the two paired samples collected from the first stool in the morning varied in the interval between positive (>250 µg/g) and negative on Day 1 and Day 2.

Røseth et al, found a similar result using daily fecal excretion of calprotectin during 3 consecutive days from patients with IBD in clinical remission and in healthy controls[127]. They also concluded that a variation in high levels of calprotectin is of little clinical concern. Tibble et al found a greater variation in single stool samples than in the total daily excretions[132]. In Crohn´s disease, and

probably also in ulcerative colitis, the variability is higher during active disease than during quiescent disease[159, 195].

The variability in apparently healthy individuals is puzzling[127, 194]. Non-specific inflammation cannot be ruled out, as histologic assessments were not carried out in these studies. Moreover, the influx of neutrophilic leukocytes might vary due to subclinical temporary events anywhere in the gastrointestinal tract, increasing the turnover of leukocytes in the mucosa. Further research is needed to clarify this issue.

In Paper II, stool samples provided monthly were analyzed for calprotectin after ileocaecal resection for Crohn's disease. The mean CV was very high (62%). As shown in Figure 9, most of the values were < 250 µg/g. To express this in a clinical context, variations of the calprotectin values <100 µg/g and <200 µg/g from the individual median value were found in 6 (43%) and 8 (57 %) of the 14 patients, respectively. In the present study, the disease activity could have changed over the 6-month period when the samples were obtained, even though the patients were in clinical remission. Moreover, there may be a difference in the CV between patients in endoscopic remission and those with endoscopic recurrence. However, in the four patients with an endoscopic recurrence, the median CV was lower than in those in endoscopic remission (40% and 69%, respectively). However, except for the anastomotic area and neoterminal ileum, disease activity in the small bowel was not evaluated.

We also investigated whether the observed variability could be explained by fecal consistency, fecal blood content or time between bowel movements (Paper I). However, neither the CV nor the SD correlated significantly with any of these variables.

In conclusion, we and others have noted variability in calprotectin levels of patients with a bowel disorder, as well as in those of healthy controls. In terms of the CV, this variability often is considerable, but to interpret correctly the CV additional descriptive data are of vital importance. To obtain stool samples from the first bowel movement in the morning seems to be a good way to decrease the impact of the variation. Furthermore, to provide another sample would be recommendable if the first one does not correspond to the clinical presentation.

5.5 Fecal calprotectin to guide treatment in ulcerative colitis

Ulcerative colitis is a chronic inflammatory bowel disorder, characterized by a relapsing-remitting clinical behavior. Pharmacologic treatment is traditionally divided into treatment of active disease and treatment to maintain remission. Conventionally, the primary aim has been to reduce the symptoms as much as possible, rather than to eliminate completely the inflammatory activity. Low-grade inflammatory activity is common in quiescent IBD and is a risk factor for a clinical relapse[29, 196]. Despite ongoing maintenance treatment, up to 50% of patients with ulcerative colitis suffer a relapse annually, with significant impact on their quality of life[26, 197, 198].

More than 10 years ago, Tibble et al presented the results of a study evaluating fecal calprotectin as a surrogate marker to predict relapse in patients with IBD[151]. In short, patients in clinical remission but with a calprotectin value >50 mg/L (an old assay was used, with current assays this is equivalent to 250 µg/g) at inclusion had a considerably higher risk of relapse during the year to come than those with a calprotectin value below that level. Since this first study was published, a number of other studies of almost identical design and one meta-analysis have been published on this topic[152, 199-205]. The results are strikingly consistent with the study performed by Tibble et al, but with varying best cut-off values and diagnostic precisions (Table 8).

Table 8. Studies evaluating fecal calprotectin as a marker to predict relapse in patients with IBD.

Author	N UC / CD	Calprotectin cut-off (µg/g)	Sensitivity(%) UC / CD	Specificity(%) UC / CD	PPV(%) UC / CD	NPV(%) UC / CD
Tibble [151]	37 / 43	250*	90	83	--	--
Costa [199]	41 / 38	150	89 / 87	82 / 43	81 / 50	90 / 83
D´Incà [200]	97 / 65	130	70 / 65	70 / 62	60 / 44	79 / 80
Gisbert [201]	74 / 89	167	69 / 69	74 / 76	35	93
GarciaSanchez [202]	69 / 66	120/200†	81 / 80	63 / 65	49 / 46	88 / 88
Kallel [203]	0 / 53	340	-- / 80	-- / 91	--	--
Yamamoto [204]	80 / 0	170	76 / --	76 / --	--	--
Naismith [205]	0 / 92	240	-- / 80	-- / 74	-- / 28	-- / 97
Mao [152]	318 / 354	167‡	77 / 75	71 / 71	--	--

UC, ulcerative colitis; CD , Crohn´s disease; PPV, positive predictive value; NPV, negative predictive value

*In the original paper, the older unit of mg/L was used (50 mg/L = 250 µg/g).

†UC and CD, respectively

‡Median value for studies included in this meta-analysis.

These studies support previous reports that normal levels of fecal calprotectin predict mucosal healing with high probability, and that mucosal healing has a strong impact on the clinical course of IBD. Contrary, elevated levels of calprotectin in quiescent disease indicate subclinical inflammation and an increased risk of symptomatic relapse. One of the most important applications of fecal calprotectin in patients with IBD is to identify subclinical inflammatory activity. A new, and readily available dimension of IBD care has emerged. Consequently, a novel treatment strategy has been proposed: to use fecal calprotectin to identify patients with IBD who are at impending risk of a flare, and to optimize treatment for those to achieve sustained remission[151, 204]. In a prospective study conducted by Maiden et al this concept was successfully implemented and the relapse rates for patients with quiescent ulcerative colitis and a calprotectin value >250 µg/g were significantly reduced by treatment with white cell apheresis[206]. Accordingly, the objective of our study was to assess targeted conventional therapy in patients with ulcerative colitis at increased risk of a flare, using regular monitoring of fecal calprotectin levels to identify these patients.

Our study was negative in terms of the primary outcome variable, the number of patients to have relapsed at Month 18. However, for the patients who accomplished a dose escalation of the 5-ASA agent, the relapse rates were significantly lower than for the patients in the control group. These results are still encouraging and further studies should be conducted on this topic.

This is the first study in patients with IBD that is designed to monitor patients over time and includes an interventional strategy when the calprotectin measurements indicate subclinical inflammation. This study was initiated in 2008 and now, 6 years later, it is exciting to read the editorial written by Patrick van Rheenen in *Inflammatory Bowel Diseases*, in which he propose a randomized trial to test this concept[207]. In the very recent trial by Osterman et al patients with ulcerative colitis in clinical remission were included and randomized to a control group or to an intervention group (i.e., dose escalation of mesalamine)[208]. The levels of calprotectin were reduced in patients who underwent the dose adjustment, whereas there was no difference in relapse rate as compared with the control group. The authors conclude that dose escalation of mesalamine reduces the concentrations of fecal calprotectin to a level associated with lower rates of relapse.

Several details of the design of a study like this have to be taken into consideration. The frequency of the calprotectin measurements, the optimal cut-off value for intervention, and a strategy for action when the calprotectin levels increase are all important factors. The easiest way to handle these issues in a clinical trial would be to define a fixed protocol that manages all the patients in the same way. In clinical practice, this might not be ideal. Instead, an individualized approach could be preferable, but remains to be evaluated.

To identify patients who are at increased risk of relapse, well before the clinical presentation of the flare, is the main problem in our study. In 10/18 patients who had a relapse in the intervention group, the calprotectin value did not reach the cut-off value before they relapsed, whereas those who had calprotectin levels above the cut-off level and who actually underwent the intervention had a reduced risk of relapsing disease. It is possible that the stool samples were obtained too infrequently, or the cut-off level was too high. In clinical practice, it would not be feasible to deliver samples and run an ELISA more frequently. However, a simple, cheap and reliable point-of-care calprotectin test that could be used at the clinic or at home by the patients themselves would be an attractive alternative for monitoring. Rapid point-of-care tests are evolving and some are already available[192, 209]. In the future, our patients with ulcerative colitis might self-monitor their calprotectin levels and adjust their own therapy, just as diabetics monitor their glucose levels and adjust their insulin doses, to avoid symptoms and complications.

The decision to use calprotectin at 300 µg/g as the cut-off for intervention was deliberately conservative. To improve sensitivity, a lower cut-off should be chosen, but to the cost of decreasing the specificity, i.e., an increasing number of patients would be over-treated. When the present study was initiated, the best cut-off values for prediction of a flare were reported to be 130-400 µg/g[151, 199, 200, 210]. In the meta-analysis done by Mao et al, the pooled sensitivity and specificity values for fecal calprotectin to predict a relapse in quiescent ulcerative colitis were 0.77 and 0.71, respectively[152]. A suggested optimal cut-off value was not presented, but the median value of included studies was 167 µg/g. However, the test performance was not as high as the authors had

expected, even though they express a potential role for calprotectin in this situation. The pooled positive and negative likelihood ratios were 2.81 and 0.31, respectively. A more clinically relevant way to describe this is to look at the positive and negative predictive values (PPV and NPV, respectively). Almost all the studies have presented a good or excellent NPV. Therefore, if a patient has a calprotectin value below the cut-off, the risk of relapse is low[200-202, 205]. On the other hand, in most of the studies, the PPV has been at best moderate, i.e., many patients with a calprotectin value above the cut-off will not relapse over the ensuing 12 months. Thus, many of these persons would be at risk of over-treatment if symptomatic relapse is the primary outcome. In our study, 43% of patients in the control group, with calprotectin values >300µg/g, did not experience a symptomatic relapse.

Our choice of cut-off value has lately gained some support. In a Dutch study, a calprotectin value > 300 µg/g in two consecutive samples was found to be the best predictor of a flare in patients with ulcerative colitis treated with infliximab[155]. Furthermore, in the STORI study, which comprised 115 patients with Crohn's disease, patients were at increased risk of relapse after discontinuation of infliximab if the fecal calprotectin at inclusion was >300 µg/g[211].

In most studies the best cut-off value for fecal calprotectin to predict the clinical course in ulcerative colitis has been 150-300 µg/g, although once again, the best option for the individual patient might be to determine the level of calprotectin in connection with mucosal healing as verified by colonoscopy. Thereafter, a strategy for treatment adjustment when the level of calprotectin is increasing should be established. We could notice, although this was not systematically studied, that many patients in remission had an individual stable level of fecal calprotectin over time and were at risk of a flare as the calprotectin level changed.

Does treatment of patients with asymptomatic ulcerative colitis, but at impending risk of a flare based on the concentrations of fecal calprotectin, represent a therapy for active disease or should it be considered as maintenance treatment? The available trials present data on treatments to induce or maintain remission, but in the present novel concept we are in between. Our aim, beyond

achieving reductions of symptoms, complications and patients' suffering, is to avoid therapy with corticosteroids. To initiate treatment with an immuno-modulating drug or anti-TNF agent in a patient with asymptomatic disease, based on a laboratory test result alone, is beyond accepted strategies. However, this might be an option if an endoscopy is performed and active inflammation is confirmed. In this type of situation, the fecal calprotectin is rather used to identify patients for a colonoscopy. In our study, a simple non-invasive strategy was preferred, so a dose adjustment of the current medication was chosen. The vast majority of the patients in the study were receiving Asacol®, for which 4.8 g has been shown to be more effective than 2.4 g to achieve treatment success in moderately active ulcerative colitis[212, 213]. In two Cochrane reports, oral 5-ASA for the induction of remission and maintenance of remission has been assessed[214, 215]. Among the various 5-ASA formulations, similar levels of efficacy and safety are described, and the benefit of using a high dose to induce remission and to maintain remission, at least for high-risk patients, is mentioned. Furthermore, in a model, inflammation-targeted, intermittent mesalamine therapy for ulcerative colitis was found to be cost-effective[216].

In our study, dose escalation was applied to 28 patients. Among these, only 8 patients subsequently suffered a relapse, and in 18 patients the calprotectin level fell from >300 µg/g to < 200 µg/g during high-dose therapy. However, the study population was too small to draw any definite conclusions from these results. Unfortunately, we were not able to recruit as many patients as planned, and this may account for the negative result in the primary outcome analysis. Another limitation of the study was the definition of a relapse. It would have been better to confirm endoscopically all relapses, even though 83% and 50% of the relapses were verified in the intervention and control groups, respectively. Furthermore, in future studies, it would be wise to exclude patients with proctitis, since our experience is that the calprotectin levels in these patients are not as stable as in patients with left-sided or extensive colitis. In the present study, only five patients with proctitis were included, three of those in the intervention group. Excluding these five patients did not change the results for the primary outcome variable (data not shown). Despite these shortcomings, the results are encouraging, and this treatment concept should be explored in a large, randomized, controlled, double-blind trial.

5.6 Fecal calprotectin to predict the clinical course for patients with newly diagnosed ulcerative colitis

Since fecal calprotectin is a predictive marker of the clinical course in patients with quiescent, established IBD, we decided to evaluate if the same applies for patients with new onset of ulcerative colitis (Paper IV). In the present study, the concentrations of fecal calprotectin at the 3-month follow-up were significantly higher in patients with relapsing disease course during the 3-year study period as compared with those with a subsequent mild disease course. In the logistic regression analysis, the calprotectin concentrations and age were the only variables that significantly predicted the clinical course.

To achieve long-term remission, mucosal healing is an important goal for therapy in IBD [30, 146, 196, 217]. In the present study, we used calprotectin as a surrogate marker of disease activity. Since normal or only slightly elevated levels of calprotectin are highly predictive of mucosal healing[184], our results support the benefit of mucosal healing in maintaining remission. The present study also underlines the importance of achieving remission early in the clinical course. Several studies have proposed early and more aggressive therapy to achieve mucosal healing and improve the clinical outcome, especially in patients with Crohn's disease[218, 219]. In ulcerative colitis the situation is somewhat different, with a clear step-wise therapy, successful in a substantial percentage of patients, and in terms of surgery, a lower risk in general. The highest risk of colectomy in ulcerative colitis has been described for patients with total colon involvement, an early need for corticosteroids, and hospitalization[25,34]. To monitor carefully these patients early in the disease course is obviously essential, and the use of fecal calprotectin is recommendable.

In contrast to previous studies exploring the ability of fecal calprotectin to predict relapse, we evaluated all patients, including those with active disease and ongoing therapy with corticosteroids. Therefore, a higher best cut-off value could be expected. A mild disease course during the first year was defined as a nonrelapsing disease, and for that period the best cut-off was 169 µg/g. This corresponds very well to recent studies (Table 8). Moreover, the best cut-off

value for the 3-year follow-up (262 µg/g) is in the same range as those in other studies. Almost 90% of the patients were in clinical remission at the 3-month follow-up. However, the median value and the IQR for all patients at that time [172 µg/g (range, 64-488)] suggest that endoscopic remission was not achieved in approximately 50% of the patients.

At what level of fecal calprotectin can we expect endoscopic remission in ulcerative colitis? In recent studies with 31-228 patients (Table 9), fecal calprotectin levels were significantly correlated with endoscopic scores (r:0.49-0.87), which is a more impressive result than those obtained with other evaluated serum and fecal biomarkers[90, 124, 126, 136, 191-193, 220].

Table 9. Correlations between the fecal calprotectin concentrations and endoscopic activity in patients with ulcerative colitis, and suggested best cut-off values for predicting endoscopic remission.

Author	No. of endoscopies	Endoscopic Activity Index	Correlation with fecal calprotectin	Calprotectin cut-off in inactive UC(µg/g)
Røseth [126]	64	Mayo score	0.57	--
D´Incà [136]	46	Mayo score	0.51	80
Langhorst [90]	42	Rachmilewitz	0.49	134
Xiang [220]	66	Sutherland	0.87	50
Schoepfer [191]	134	Rachmilewitz	0.83	50
D´Haens [124]	39	Mayo score	0.62	250
Schoepfer [193]	228	Baron score	0.82	57
Lobatón [192]	146	Mayo score	0.74	160

UC, ulcerative colitis

In the study performed by D´Haens et al, the best correlation between calprotectin and endoscopy was found when a combination of extent and severity of disease was used[124]. In the largest study, Schoepfer et al evaluated 228 patients with colonoscopy using a modified Baron Index[193]. Comparing several noninvasive markers with endoscopic activity, fecal calprotectin performed the best (r=0.82). With a very low cut-off value (57 µg/g), the sensitivity and specificity to detect active disease were 91% and 90%, respectively, and the area under the receiver operating curve (AUC) was 94%. In this study, patients with proctitis were excluded, making it difficult to compare the results with those of other studies. However, in clinical practice, the need for a full colonoscopy for patients with proctitis can be argued, making this study more appropriate than others. In a meta-analysis, Lin et al have suggested a cut-off value for active disease of 250 µg/g, with a pooled sensitivity of 80% and

specificity of 82%[190]. Accordingly, at this cut-off value, 18% of the patients will be identified with active disease despite having a normal endoscopy and 20% of the patients will be missed. The authors discuss the possibility of overestimating the accuracy of calprotectin and the cut-off value due to a large proportion of the patients exhibiting active disease. Furthermore, compared to Crohn's disease, calprotectin appears to have superior ability to evaluate disease activity in ulcerative colitis.

Let us consider the results presented in Paper III and Paper IV. Patients with new onset of ulcerative colitis and in clinical remission after the initial treatment, but still having increased values of fecal calprotectin, are at increased risk of relapsing disease course. Thus, early in the clinical course, monitoring with calprotectin can help us to identify many of these patients and a strategy for therapy to reach the goal i.e., mucosal healing, can be established. Furthermore, the possibility to identify at an early stage those patients who are at risk of relapsing disease course can be the basis for constructive discussions to motivate and optimize compliance with therapy. Further investigation might be an option if initiation of additional therapy is considered. Thereafter, frequent monitoring should be carried out until calprotectin falls below a predetermined cut-off value, after which monitoring is performed on a regular basis with ongoing maintenance therapy. Theoretically, this is an attractive model[207]. In a very recent study from Barcelona, 64 patients with ulcerative colitis were thoroughly followed-up every 3 months during 1 year. The fecal calprotectin value at each visit predicted significantly a relapse during the subsequent 3-month period, but the sensitivity was low, although the specificity was good[221]. The level of specificity in our study (Paper IV) was also good, although the sensitivity was insufficient, i.e., about 36% of the patients who relapsed during the first year were not identified by the test using the stated cut-off.

The results of our studies are not sufficiently robust to recommend a new treatment strategy based on the levels of calprotectin. Nevertheless, the data are promising and further research is warranted.

5.7 Fecal calprotectin and shortcomings

In several studies, fecal calprotectin has been the best biomarker to assess disease activity, as compared with other biomarkers and clinical indices. However, some shortcomings have to be mentioned. Fecal calprotectin is not disease-specific, and elevated levels can be found in any gastrointestinal disease that involves mucosal infiltration of neutrophils. Accordingly, in infectious gastroenteritis, diverticulitis, ischemic colitis and neoplasms, the calprotectin levels are increased. Elevated levels can also be found in patients with upper gastrointestinal diseases[222]. In studies that have included patients with cancer, elevated levels of fecal calprotectin have been found constantly[125, 145, 157, 169, 223]. In two studies including 149 and 80 patients with colorectal cancer, the median calprotectin levels were 372 µg/g and 205 µg/g, respectively[157, 223]. However, very high levels of calprotectin (>1000 µg/g) are almost exclusively found in patients with active colitis, especially IBD[145].

Increased levels of calprotectin have likewise been found in patients who are taking non-steroidal anti-inflammatory drugs (NSAID), which cause inflammatory lesions throughout the gastrointestinal tract[224-226]. It appears as though the severity of the NSAID-induced enteropathy, and thereby the concentrations of fecal calprotectin, are independent of the type of NSAID being taken. However, in a study from Gothenburg, increasing levels of fecal calprotectin were detected with increasing frequency of NSAID intake in patients with ankylosing spondylitis[227]. In the study by Meling et al, after a wash-out period of 3 weeks after completing the NSAID therapy, the fecal calprotectin levels had returned to baseline levels[224]. However, low-dose aspirin does not induce an increase in fecal calprotectin concentrations[228].

Different life-style factors, such as obesity and physical inactivity, as well as the use of proton pump inhibitors and stool consistency have been reported to influence the fecal calprotectin levels[168, 229]. The clinical consequence of this is probably not significant. An increase in calprotectin levels has also been discussed when stool samples are collected at time of a respiratory tract infection or when the samples are contaminated with blood, i.e., during nasal

bleeding, gastrointestinal bleeding or menstruation[125]. However, a considerable amount of blood in the feces would be required to increase to any significant extent the concentrations of calprotectin[161, 169].

High levels of fecal calprotectin are commonly seen in young healthy children, and in infants, the levels are 10-fold higher than the normal level for adults[230]. In children aged 4 years and older, the most commonly proposed cut-off level for adults (50 µg/g) can be used[231]. An increase in fecal calprotectin concentration with age has been reported in adults, although the clinical significance of this is probably low[168].

The variability of fecal calprotectin levels has to be mentioned, although it has been discussed previously.

In some studies, the precision in terms of sensitivity and specificity is poor, while in others it is extremely good, resulting in meta-analyses with at least modest or often good results. The problem with the different cut-off values has been mentioned. The results may have been influenced by both the inclusion of a too high proportion of patients with clinically active disease, and a high number of studies from tertiary centers. To explore only patients with quiescent disease would be of greater clinical interest to establish a relevant cut-off that would distinguish between patients with mucosal healing and those with subclinical inflammation. It is also noteworthy that in two very recent, large studies, the cut-off values for active disease were set at <100 µg/g[174, 193]. This may indicate that the definitions of endoscopic remission have changed over time. However, it remains difficult to compare the studies, as study populations differ and different endoscopic activity scores have been used.

Recently, discrepancies between different commercial assays for calprotectin have been highlighted[232, 233]. This is unfortunate because it hinders accurate comparisons between studies and between different laboratories. It is crucially important that the manufacturers of commercially available assays agree on a standardization.

Finally, fecal calprotectin is a rather novel marker of disease activity, not perfect, but so far the best marker of disease activity in patients with IBD.

6 SUMMARY AND CONCLUSIONS

- Calprotectin levels measured from small, randomly obtained samples from the stool are useful in clinical practice, as the correlation between two samples from the same stool is good, suggesting an even distribution of calprotectin in feces.

- The variability of fecal calprotectin is considerable in active ulcerative colitis. To interpret the clinical consequences of the variability, additional descriptive statistics is necessary.

- To reduce the consequences of the variability and to reduce the risk of a misleadingly low calprotectin value, it is recommended to obtain samples from the first stool in the morning.

- It is not advisable to store stool samples at room temperature for more than 3 days before analyzing it for calprotectin.

- Patients with ulcerative colitis do not find it burdensome to provide stool samples. Adequate equipment for this purpose is preferred.

- The concentrations of fecal calprotectin do not distinguish between patients with endoscopic recurrence 1 year after ileocaecal resection for Crohn's disease and those without. The great majority of patients with low values (< 100 µg/g) do not have an endoscopic recurrence and in those a colonoscopy might be avoided.

- Fecal calprotectin in patients treated for their first attack of ulcerative colitis is predictive of the further clinical course. Careful follow-up and therapeutic considerations for newly diagnosed patients with increased levels of calprotectin are justified.

- In asymptomatic patients with ulcerative colitis, identified with a calprotectin level >300 µg/g, dose escalation of a 5-ASA agent significantly reduced the relapse rate as compared with the corresponding patients in a control group. However, the overall risk of relapse was not different in these patients providing stool samples every month. These results are encouraging and further trials are proposed.

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Hélène, I love You.

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9 APPENDIX

9.1 Appendix A

Dag 1 Datum

Ange tid, konsistens samt ev blod för varje tarmtömning.

Toabesök	**Klockan**	**Bristol skalan**	**Blod**
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2			
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9.2 Appendix B

Enkät

* För att undvika att avföringen och därmed provmaterialet blandas med vatten har Du använt ett uppsamlingspapper i toalettstolen. Hur upplevde Du att utföra Dina behov med denna anordning i toalettstolen?

Mycket besvärligt Besvärligt Acceptabelt Inga problem

* Hann Du fästa upp uppsamlingspappret eller var trängningarna så akuta att det inte fanns tid för det?

Hann aldrig Hann ibland Hann för det mesta Hann alltid

* Hur upplevde Du själva provtagningen med avsedd sked?

Mycket besvärligt Besvärligt Acceptabelt Inga problem

* Hur upplevde Du momentet att lossa uppsamlingspappret och att spola ner det?

Mycket besvärligt Besvärligt Acceptabelt Inga problem

* Har Du tagit avföringsprov med liknande sked tidigare?

Ja Nej

* I så fall, användes uppsamlingspapper vi de tillfällena?

Ja Nej

* Vilken provtagningsform föredrar Du?

Med uppsamlingspapper Utan uppsamlingspapper

* Egna kommentarer:

9.3 Appendix C

Bristolskalan över avföringsformen	
Typ 1 	Separata hårda klumpar likt nötter (svåra att få ut).
Typ 2 	Korvformad men med klumpar.
Typ 3 	Likt en korv, men med sprickor på ytan.
Typ 4 	Likt en korv eller orm, smidig och mjuk.
Typ 5 	Mjuka klumpar med skarpa kanter (enkla att få ut).
Typ 6 	Fluffiga bitar med trasiga kanter.
Typ 7 	Vattnig, inga fasta bitar. Enbart vätska.