POST-OPERATIVE CROHN'S DISEASE: CAN NON-INVASIVE FAECAL MARKERS PREDICT POST-OPERATIVE COURSE OF CROHN'S DISEASE?

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS:

CD	Crohn's disease
UC	Ulcerative colitis
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
FC	Faecal calprotectin
FL	Faecal Lactoferrin
CRP	C-reactive protein
WCC	White cell count
ASCA	Anti-Saccharomyces cerevisiase antibodies
ANCA	Anti-neutrophil cytoplasmic antibodies
ESR	Erythrocyte sedimentation rate
HBI	Harvey Bradshaw activity index
CDAI	Crohn's disease activity index
ASA	Aminosalicylate
CARD15	Caspase-activated recruitment domain
NOD2	Nucleotide binding oligomerization domain 2
GWAS	Genome wide association studies
NaOH	Sodium hydroxide
ELISA	Enzyme linked immunosorbent assay
IL23	Interleukin 23 receptor
ATG16L1	Autophagy related 16 like1 gene
IRGM	Immunity-related guanosine triphosphatase: human homologue of the mouse

Abstract:

Faecal calprotectin or lactoferrin can identify post-operative recurrence in Crohn's disease.

Background:

Identifying Crohn's disease recurrence in symptomatic patients after ileocaecal resection is difficult as symptoms may reflect the effect of surgery rather than active disease. The aim of this study was to evaluate faecal concentrations of granulocyte degradation products (faecal calprotectin and faecal lactoferrin) in this post-operative setting.

Methods:

A post-operative cohort of 104 patients (median follow up of 24 months) provided a single stool sample. A second cohort of 13 patients was followed prospectively for 1 year with regular faecal calprotectin (FC) and faecal lactoferrin (FL) measurements. Faecal measurements were compared with symptom diaries, the Harvey Bradshaw Index (HBI), endoscopic examination, C-reactive protein (CRP) and platelet measurement.

Results:

Both faecal calprotectin and faecal lactoferrin correlated significantly with Harvey Bradshaw Index ($r = 0.532$, $P < 0.001$, $r = 0.687$, $P < 0.001$ respectively). Twenty eight patients with severely clinically active disease had high mean (s.e) levels of faecal calprotectin (661.1(119.1) µg/g) and

faecal lactoferrin (116.6(32.2) µg/g); and forty three patients with clinically inactive disease had low levels of faecal calprotectin (70.2(27.1) µg/g) and faecal lactoferrin (5.9(2.4) µg/g). In patients with mild to moderately clinically active disease, faecal calprotectin and faecal lactoferrin identified individuals with and without recurrent inflammatory disease. In the uncomplicated course, both markers (faecal calprotectin and lactoferrin) normalized within 2 months. Faecal markers were more accurate at predicting clinical disease activity than C-reactive protein, platelet count or endoscopic appearance.

Conclusion:

Faecal calprotectin and faecal lactoferrin are non-invasive tests that can help to identify disease recurrence in symptomatic post-operative patients [1].

Chapter 1

Introduction

1.0 Rationale for research:

Crohn's disease is a serious and debilitating chronic disease affecting 1:1000 of the adult population. Onset of the disease is often in childhood or early adulthood. Crohn's disease is notoriously variable in presentation and behaviour. At the severe end of the spectrum there is a substantial impairment of the quality of life and studies have suggested 15-20% of Crohn's disease patients will be rendered unable to work by their disease. The study of post-operative events in Crohn's disease represents a unique opportunity to identify the disease recurrence in post-operative patients.

As there is no single gold standard test, endoscopy and histology is used for detecting and quantifying gastrointestinal inflammation. However this method is invasive, expensive and not well tolerated by patients [2]. In the search for less-invasive methods for diagnosis and monitoring of inflammatory bowel disease, faecal calprotectin and lactoferrin show promise as simple, non-invasive, inexpensive sensitive and specific parameters to detect gastrointestinal inflammation [3-6]. Other various biological and serological markers such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Anti-saccharomyces cerevisiase (ASCA) and anti-neutrophil cytoplasmic antibodies (ANCA) have low sensitivity and specificity for gastrointestinal inflammation and poorly correlate with disease activity [3, 7, 8].

Smokings, perforating behaviour, ileal or ileocolonic location and female sex have all been associated with a tendency to early and aggressive recurrence after resectional surgery. Recognition of these factors can provide a means to stratify risk broadly within a population, but prediction of recurrence in an individual remains poor. After resection surgery patients with Crohn's disease often develop symptoms suggestive of relapse. It is hard to establish clinically whether these symptoms are due to recurrence or rapid gut transit, as a consequence of adhesions after surgery, salt malabsorption or functional bowel disorder.

The ideal assessment of the post-operative patient would be a reliable, non-invasive marker of recurrent disease that can be monitored, correlating both with symptom relapse and mucosal ulceration. Faecal concentration of granulocyte degradation products (faecal calprotectin and lactoferrin) may therefore offer a suitable method for assessment of disease activity after ileocolonic resection.

Previous research has shown both faecal calprotectin and lactoferrin are able to differentiate organic from functional disease, simple to use, reliable, inexpensive and sensitive for gastrointestinal inflammation and non-invasive. The clinical usefulness of faecal calprotectin and lactoferrin in the post-operative setting has not been prospectively established.

Quantitative measurement of faecal calprotectin and faecal lactoferrin may offer a valuable method as non- invasive markers in assessing the symptomatic post-operative patient in Crohn's disease.

To investigate this above hypothesis this study had the following aims:

1) To determine whether faecal calprotectin and faecal lactoferrin measurements in symptomatic post-operative Crohn's patient can differentiate those with early disease relapse from those with late relapse.

2) To compare faecal calprotectin and faecal lactoferrin with other measurements of inflammatory activity such as C-reactive protein, white cell count, platelet count, clinical disease activity index (HBI) and endoscopic finding.

3) To identify the heterogeneous group of patients (those with mild to moderate disease activity) using CRP, white cell count and faecal calprotectin and lactoferrin and there correlation and also looking at smoking status and maintenance therapy with these markers.

4) To determine how faecal calprotectin and faecal lactoferrin correlate with each other after resection.

5) To determine the immediate post-operative course of faecal calprotectin and faecal lactoferrin after ileocaecal resection.

6) To determine whether these faecal markers could demonstrate inflammatory activity and identify post-operative disease recurrence.

7) To identify if there is a difference in this two markers among uncomplicated and complicated group (with post-operative complications

like anastomotic leak, intra-abdominal collection, wound infection and disease recurrence) of patients in immediate post-operative setting.

This thesis initially gives all background information about Crohn's disease including post-operative setting and faecal markers (literature review) in introduction chapter. Second chapter has details of the experiments used for measuring the concentration of CRP, white cell count, platelet count and faecal calprotectin and faecal lactoferrin. The results chapter (both cross-sectional and longitudinal study) addresses the aims of the study. Lastly the discussion chapter gives the conclusions of the study along with examining the literature critically and future prospects in the use of faecal calprotectin and lactoferrin as non-invasive markers in post-operative setting.

1.1 Introduction:

Crohn's disease is a chronic relapsing disease which affects any part of the gastrointestinal tract from mouth to anus, but is most often restricted to small bowel, in particular the terminal ileum (figure 1.1). It is characterised by patchy transmural inflammation. It can involve perianal skin lesions or multiple areas of small bowel with normal bowel (skip lesions) in between or may involve the whole of the colon or small bowel extensively. It may be defined by location (terminal ileal, ileocolic, colonic, or upper gastrointestinal) or by disease pattern (inflammatory, fistulating or structuring). The involved bowel may be narrowed, thickened, with deep ulcers and fissures in the mucosa: giving a cobble-stone appearance (figure 1.2).

1.2 History:

What we call now Crohn's disease was described in detail by a Glaswegian surgeon, Kennedy Dalziel in the British Medical Journal (1913) as chronic intestinal enteritis. In the 20th century between 1914 and 1932, lots of articles describing chronic intestinal enteritis were written. Finally in 1932 the definitive paper describing the condition as regional ileitis was published and named after first author: Burrill B Crohn, a Mount Sinai Hospital physician [9, 10].

1.3 Epidemiology:

The incidence of Crohn's disease is 5-10 per 100,000 per year with a prevalence of 50-100 per 100,000. It is generally a disease of young people with a peak incidence between 10 and 40 years. But it may affect people of any age group although 15% of people are over the age of 60 years at diagnosis. There are marked differences between ethnic groups with some (such as Ashkenazi Jews) having a particularly high incidence [11-14].

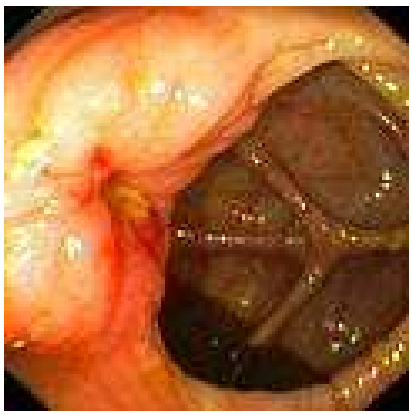

Figure 1.1 - Endoscopic picture of Ileocolic Crohn's disease.

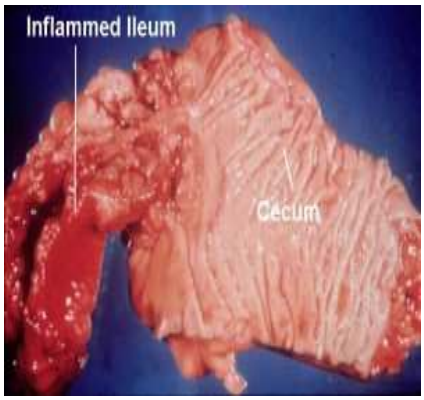

Figure 1.2 - Crohn's disease pattern showing narrowing, ulceration, fistulae-cobble stone appearance (image from odlarmed.com).

1.4 Pathogenesis:

The aetiology of Crohn's disease remains unknown. The consensus is that Crohn's disease is a response to environmental triggers like infection, drugs or other agents in genetically susceptible individuals. Epidemiological studies have considered diet, drug, vaccination history, seasonal variation, water and social circumstances for the pathogenetic mechanism of Crohn's disease. The gut/environmental interface includes work on the luminal bacteria, epithelial glycocalyx, mucus, epithelial remodelling and barrier function, and lastly immune and epithelial interaction. The inflammatory factors have been examined through inflammatory mediators, lymphocyte trafficking, cell signalling pathways, cytokine profiles, and interaction between stromal and immune cells [15].

The genetics approaches included linkage studies, candidate gene studies and recently Genome Wide Association Studies (GWAS). More than fifty confirmed inflammatory bowel disease genes have now been identified. Inflammatory bowel disease is associated with variation in CARD15, IL23, IL12B, JAK2 and STAT3, these are associated with inflammatory bowel gene susceptibility consistent with newly described role of IL23 signalling and Th13 cells in disease pathogenesis [16]. In Crohn's disease NOD2 and autophagy genes ATG16L1 and IRGM are defective in different aspects of bacterial handling [17]. Wide genome associated studies have been successful in defining the genetic architecture and identifying susceptible

genes involved in Crohn's disease. This will help to explore more in detail the natural history of this complex disease in future.

1.4.1 Environmental factors:

Many environmental factors have been proposed as mentioned above in Crohn's disease susceptibility. These factors include are diet, domestic hygiene, other microbial or viral antigens and smoking.

1.4.2 Diet:

Many studies have proposed that diet may play a role in Crohn's disease, either through infectious agents in food or via immune response to dietary antigens. Milk has been implicated to be involved with Crohn's disease, in particular the Mycobacterium avium subspecies paratuberculosis. Although Mycobacterium paratuberculosis is a causative agent in Johne's disease, which is a form of inflammatory bowel disease in deer, sheep, cattle and monkeys: this theory has not been confirmed [18].

1.4.3 Domestic Hygiene:

Better hygiene during childhood may be associated with more risk of developing inflammatory bowel disease. This has been proposed to work in two ways: either these individuals have less antigen exposure in the form of bacteria or virus in turn less immune resistance or immune tolerance requires continuous exposure to danger. Secondly, it has been proposed that immune tolerance requires continuous exposure to danger. According

to these hypotheses inadequate exposure to environmental stimuli in early childhood may lead to immune intolerance. This may suggest autoimmunity among less exposed or clean individuals in later life [19, 20].

1.4.4 Microbial or viral antigens including vaccines:

Bacteria and viruses may trigger inflammatory bowel disease. Absence of inflammation in various genetic models of colitis such as animals in germ free conditions show a role for intra-luminal bacteria in disease pathogenesis [21]. Effective therapies and extensive use of antibiotics are used in treatment of Crohn's disease [22]. Either live or attenuated viral vaccines may trigger inflammatory bowel disease [23, 24]. The peptides in vaccines may mimic self antigens in the bowel leading to an autoimmune response and Crohn's disease. This relationship of vaccines and infection in IBD remains controversial [19].

1.4.5 Smoking:

One of the most intriguing environmental factors found to be associated with inflammatory bowel disease is smoking, both from its potential relevance to disease expression and from clear differences found between its frequency in Crohn's disease and Ulcerative colitis. A strong correlation between smoking and inflammatory bowel disease was reported in 1982 by Harries et al [25]. In 1990, It was demonstrated that there is an association between smoking and the more severe course's of Crohn's disease [26]. In this study group of 174 Crohn's disease patients who underwent surgery it was shown

that over 10 years smokers have 29% increased risk of having a further surgery than non-smokers. Five and ten year recurrence rates were significantly higher in smokers (36% & 70%) than in non-smokers (20% & 41%) [26]. In 1994 a multivariate analysis conducted to examine predictive factors for clinical, endoscopic and surgical recurrence after surgery found smoking to be an independent factor for all three types of recurrence. The six year disease free recurrence after surgery was 27% for smokers, 41% for ex-smokers and 60% for non-smokers [27].

Effects of cessation of smoking were described by Cosnes et al on short term course shown in figure 1.3 [28]. The risk of flare up in the smoking quitters did not differ from that in non-smokers and was less than in continuing smokers ($p < 0.001$). Cosnes et al concluded Crohn's disease patients who stop smoking for more than one year have a benign course than if they had never smoked. Lindberg, in a study of 221 patients came to the conclusion that in heavy smokers the risk of surgical intervention at five years and ten years was higher. Also in this particular study group he found smokers to have an increased risk of perforating complications when compared to non-smokers [29].

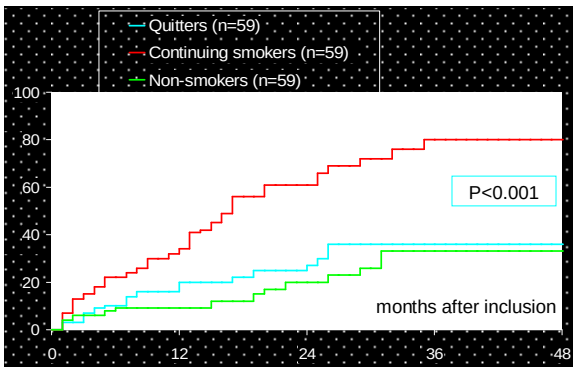

Figure 1.3 - Effect of stopping smoking on the post-operative course of Crohn's disease [28].

In 1998 a study conducted by Timmer et al found that at forty-eight weeks after surgery for Crohn's disease the relapse rate was 30% in non-smokers compared to 53% in smokers. Ex-smokers had a similar relapse rate (35%) to non-smokers [30]. In a study by Yamamoto et al in 1999 demonstrated the five and ten year recurrence rate after ileocaecal resection for Crohn's was 35% and 55% for smokers compared to 19% and 36% for non-smokers respectively(p=0.007) [31]. In another study done by the same group it was found that the recurrence rate for Crohn's colitis at 5,10,and 15 years was 25%,46%, and 52% respectively for smokers and 11%,15%, and 18% respectively for non-smokers. Smoking was found to be an independent significant predictor for poor outcome after surgery for Crohn's colitis following multivariate analysis [32].

Another study done by Ryan et al in 2004 examined the impact of stopping smoking on re-operation for Crohn's disease recurrence after surgery. They found that patients who stopped smoking were less likely to have required (undergone 1, 2, and 3) re-operations. This data suggests that patients who stop smoking reduce the risk of re-operation for recurrence of Crohn's disease [33]. On the contrary, there were two studies which didn't find any relevant correlation between smoking and the recurrence rate after surgery for Crohn's disease. Both the studies had a small numbers of patients (40 patients with CD) and detailed data regarding smoking was not included [34, 35].

In conclusion, it appears that smoking has a great impact on increasing the risk of recurrence after surgery for Crohn's disease, especially in heavy smokers and women. From the studies published, stopping smoking is correlated with a lower recurrence rate compared with smokers. Education and encouraging patients to stop smoking is important in the management of Crohn's disease as most patients are unaware of the risks involved with smoking on their disease [36-40]. How smoking effects Crohn's disease is still unclear. Potential factors involved include gut permeability, gut mucosal composition, perturbation in eicosanoid production, and immune modulation as reported in a few studies [11, 41-44]. Correlation between the faecal markers and smoking status has been examined in the cross-sectional results chapter of this thesis.

1.5 Clinical features of Crohn's Disease:

Symptoms of Crohn's disease are heterogeneous, typically including abdominal pain, diarrhoea, and weight loss. Systemic symptoms include malaise, anorexia, and fever. Crohn's disease patients may also present with symptoms of intestinal obstruction due to strictures, fistulae and abscesses. Surgery is not curative in Crohn's disease patients and the management is aimed at minimising the impact of disease. About 50% of the patients diagnosed with Crohn's disease undergo surgical treatment within the first 10 years of the disease. 70-80% will require surgery at some point in life [45]. The mortality rate of Crohn's disease is greatest in the first two years after diagnosis and in those with upper gastrointestinal disease [46].

1.6 Diagnosis:

The diagnosis of Crohn's disease can be challenging as there is no single gold standard. Colonscopy and biopsy is approximately 70% effective in diagnosing the disease, with further test being less effective. Disease in small bowel is particularly difficult to diagnose as a traditional colonscopy allows access to only the colon and lower portion of small bowel, introduction of capsule endoscopy aids in endoscopic diagnosis. White blood cell scan have shown to be effective in detecting the location of active disease but this procedure is expensive, time consuming and involves radiation. So the diagnosis is reached upon by a combination of clinical

evaluation, and diagnostic investigations including endoscopic, histological, radiological, and/or biochemical investigations.

1.6.1 History and Examination:

A detailed history must be obtained including the onset of symptoms, any recent travel, intolerance to any food, contact with patients with enteric illnesses, family history, past medical history of appendicectomy, medication (antibiotics and non-steroidal anti-inflammatory drugs), and smoking. On examination, one must observe the general well being of the patient, baseline observations, abdominal tenderness or distension, any palpable masses, perianal inspection, oral inspection, and digital rectal examination. One must look for extra intestinal manifestations like uveitis, episcleritis, arthritis, erythema nodosum and pyoderma gangrenosum as well.

1.6.2 Laboratory Investigations:

Routine laboratory investigations are C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and full blood count (FBC). Stool sample must be sent for microbiology assay for clostridium difficile toxin.

1.6.3 Procedures to establish the diagnosis:

To establish the diagnosis ileocolonoscopy and terminal ileum biopsies are used, also they help evaluate the extent and provide microscopic evidence of Crohn's disease throughout the colon. Other investigations to evaluate the extent of disease are small bowel follow through, small bowel enema,

transabdominal ultrasound, computed tomography-enterolysis and magnetic resonance imaging. In certain patients admitted as emergency with right iliac fossa abdominal pain, laparoscopy for diagnosis has identified CD. Wireless capsule endoscopy may have a role for small bowel imaging.

1.6.4 Histopathology:

Microscopic features to look for in diagnosing Crohn's disease are focal (discontinuous) chronic inflammation (lymphocytes and plasma cells), patchy chronic inflammation, focal crypt irregularity, and granulomas. Transmural inflammation, aggregated inflammatory pattern, transmural lymphoid hyperplasia, sub mucosal thickening, fissures, sarcoid granuloma (including in lymph nodes), and abnormalities of the enteric nervous system are the microscopic features seen in the surgical specimen.

1.7 Management:

There are guidelines for the management of Crohn's disease that have been published and recommended by British Society of Gastroenterology [47]. The following medical and surgical treatments are a summary from these guidelines. The treatment of Crohn's disease depends on the disease activity, site of disease (ileal, Ileocolic, colonic, other), and behaviour of disease (inflammatory, stricturing, fistulating), response to previous medical therapy and side effects of medications. Patient's involvement should be actively encouraged in decision making.

1.7.1 Medical management:

The general principles in the management are to consider the site (ileal,ileocolic,colonic), pattern (inflammatory,stricturing,fistulating) and activity of the disease before treatment decisions are made in conjunction with the patient. An alternative explanation for the symptoms other than active disease should be considered (such as bacterial overgrowth, bile salt malabsorption, fibrotic stricture) and disease activity confirmed (usually CRP) before starting steroids. Patients with Crohn's disease have many investigations over their lifetime and imaging should not be repeated unless it will alter management or a surgical decision depends on the result. In mild ileocolonic disease high dose mesalazine may be sufficient as initial therapy. Patients with moderate to severe or those with mild disease that has failed to respond to oral mesalazine, oral prednisolone 40mgs daily is appropriate. Prednisolone should be reduced gradually over a period of 8 weeks. Active ileal or ileocolonic Crohn's disease should be treated with high dose of budesonide, corticosteroids & nutritional therapy. Surgery is tailored depending on disease severity and patients view [48-51]. Systemic corticosteroids are used in treating severely active localised ileocaecal Crohn's disease [52]. Azathioprine 1.5-2.5 mg/kg/day or mercaptopurine 0.75-1.5 mg/kg/day may be used in active Crohn's disease as adjunctive therapy and as a steroid sparing agent or Patients who relapse, azathioprine/mercaptopurine should be added (or, if intolerant, methotrexate is considered) [53-55]. In fistulating and perianal disease, the initial management aim should be to treat active disease and sepsis, defining the

anatomy, supporting nutrition and potential surgery. At times MRI and examination under anaesthetic are particularly helpful. Metronidazole 400mgs three times daily is appropriate first line of treatment for simple perianal fistula. If distal obstruction and abscess has been ruled out azathioprine or mercaptopurine are effective for enterocutaneous fistulae. Infliximab infusions at 0,2 and 6 week is reserved for those patients whose perianal or enterocutaneous fistulae are refractory to other treatments and used as a part of a strategy that includes immunomodulation and surgery. Though surgical options such as seton drainage, fistulectomy and use of advancement flaps in combination with medical treatment is appropriate for persistent or complex fistulae [56-58]. In maintenance of remission patients who smoke should be offered help to stop and immunomodulation with azathioprine, mercaptopurine or methotrexate is usually appropriate as steroids are withdrawn.

1.7.2 Surgical management:

Surgery should be advised for Crohn's disease not responding to intense medical therapy. The decision to operate is best taken by the gastroenterologist and colorectal surgeon in conjunction with the patient. Surgery for Crohn's disease is undertaken for symptomatic rather than asymptomatic, radiological disease because the disease is pan-enteric and usually recurs following surgery. The general principles are preoperative counselling and marking of stoma sites. The procedure of choice in acute fulminant Crohn's disease is subtotal colectomy leaving a long rectal stump,

either incorporated into the lower end of the abdominal wound or mucous fistula. Resection should be limited to macroscopic disease. Primary anastomosis should not be performed in presence of malnutrition or sepsis. Crohn's disease has two different forms, a perforating (acute free perforation, subacute perforation with abscess formation and intestinal fistula formation) and non-perforating (intestinal obstruction, haemorrhage, medical intractability and toxic dilation) [59].Localised ileocaecal Crohn's disease with obstructive symptoms can be treated with primary surgery [60]. Abdominal abscesses are managed with antibiotics and percutaneous or surgical drainage followed by delayed resection if necessary [61]. Stricturoplasty is performed when the length of small bowel stricture is <10 cm but is not recommended for colonic Crohn's disease. Endoscopic dilatation of the strictures is preferred for the management of accessible short strictures [62, 63].

1.8 Literature review-

Bibilographical searches were performed in MEDLINE electronic database up to September 2009 looking for the following keywords words (all fields): inflammatory bowel disease or Crohn's disease or Ulcerative Colitis, postoperative Crohn's disease or surgery for Crohn's disease, faecal markers, faecal calprotectin or faecal lactoferrin, clinical indices. Further articles were identified by use of the related-articles function in PubMed. A manual citation search of references given in the retrieved articles on the subject was also performed. Only articles published in English language were chosen.

1.8.1 Postoperative Crohn's disease- Background:

Crohn's disease is a chronic inflammatory bowel disease with complex pathophysiology. Its heterogeneous clinical presentation has challenged clinicians due to its inconsistency in patient presentation, leading to difficulties in predicting the clinical course [64]. Over three quarters of patients diagnosed with Crohn's disease require surgery and about one third of these patients will require further surgery when followed long term [65].Despite recent progress in medical therapy, surgery is still necessary for the complications of Crohn's disease and bowel resection and has resulted in better outcomes for many patients. However multiple operations can risk short bowel syndrome and result in difficult procedures due to adhesions [66].

Numerous pathological and demographical factors have been investigated for their supposed influence on recurrence of Crohn's disease [67]. The only assessed environmental risk factor seems to be smoking to identify recurrence after surgery, as described in detail in earlier section [38].Several factors like gender, family history of Crohn's disease, presence of granulomas, obstructing, fistulising nature of disease, length of surgical margins, site and number of sites involved, type of operation and number of anastomosis did not show any definite factor to be implicated in recurrence [68-70]. A number of studies have revealed several risk factors for post operative recurrence; however the results are not similar because of incompatible patient populations and clinical manifestations [67, 71, 72]. Despite these studies post-operative recurrence remains clinically important, but poorly understood.

1.8.2 Indications for surgery:

Two large studies found the cumulative probability of surgery of 78% at 20 years from the diagnosis of Crohn's disease. In these studies 66% of patients had limited small bowel Crohn's disease requiring surgery compared to 58% for colonic Crohn's disease [73, 74]. The most common reason for surgery has traditionally been for complications of Crohn's disease like intestinal obstruction, strictures and perforation (22% to 45%) or medical intractability (16% to 68%) [66, 73, 75].

1.8.3 Recurrence after surgery:

Recurrence after surgery is usually at the anastomotic site after long term follow up of Crohn's disease [76]. Only 20% of patients develop clinical symptoms compared to endoscopic recurrence of 73% after surgery at 1 year follow up and at 3 years 34% and 85% (respectively) [77]. This study demonstrated that the development of one form of recurrence correlates poorly with other. Two large studies have shown that primary ileocolic surgery for ileocolic disease is generally associated with an increased reoperation rate compared to isolated ileal or colonic disease [73, 74]. These studies showed 53% (ileocolic disease), 45% (colonic disease) and 44% (ileal disease) reoperation rate after follow up of thirteen years. A few small studies have shown no difference in reoperation rate [78, 79]. Most of the studies consistently conclude that about 50% of patients who have undergone ileocolic resection for Crohn's disease will require further operation within 10 years [75, 80].

Some studies have identified that more extensive disease preoperatively is associated with increased risk of recurrence for both symptoms [68, 81, 82] and reoperation rate [83]. A few studies have suggested that patients with Crohn's disease who present with bowel perforation are more likely to have reoperation [70, 84, 85] on the contrary another study [86] suggested that perforating nature of disease does not carry a greater risk of recurrence. Stenosing and fistulising Crohn's disease patients of ileocaecal site have no

difference in recurrence rate after bowel resection after 9 year follow up [87].

Bowel sparing surgical policy, which involved resecting only the macroscopic diseased bowel [88, 89], was found to have reasonable recurrence rates compared to extensive bowel resection or presence of microscopic disease at surgical margins [90]. Stricturoplasty for Crohn's disease patients for small bowel stricture<10 cm has shown to have a similar postoperative recurrence rate to resection [63, 91, 92].

Further clinical studies based on specific anastomotic techniques have had variable results. Stapling techniques results have been shown to have increased recurrence after resection as a result of localised ischaemic injury [82, 93]. Other studies have shown no difference between hand sewn or stapled anastomosis [94, 95]. No randomised trials exist and due to different sites of disease, follow up time, and specific techniques, so it is not easy to make a firm conclusion. Clinical, endoscopic and histological criteria are all used in post-operative setting but their correlation is imperfect [73, 96-98] and the standard clinical assessment tools for measuring CD activity are not necessarily useful in post-operative setting [97].

1.8.4 Post-operative complications:

2% of Crohn's disease patients develop post-operative complications in the form of leaks or dehiscence even with bowel sparing surgery or stricturoplasties. These complications are associated with pre-existing impaired nutritional state, steroid therapy, existing sepsis or obstruction and multiple previous surgeries. Two studies [99, 100] have shown that development of post-operative complications is associated with increased recurrence rate and another study [101] has shown no relationship between increased recurrence with post-operative complications.

1.8.5 Medical prophylaxis against post-operative recurrence:

The effect of 5-aminosalicylate in recurrence post-operatively in Crohn's disease is controversial. If there is a beneficial effect it is likely to be small [102, 103]. Two randomised control studies [104, 105] have reported that nitroimidazole antibiotics are effective but dosage and duration remain questionable. The use of immunosuppressant agents like azathioprine, 6-mercaptopurine and antitumor necrosis (TNF) alpha antibody therapy have been shown to produce mucosal healing in the treatment of post-operative disease recurrence [106, 107]. Conversely, these immunosuppressants do have side-effects and are therefore only desirable for targeted therapies with proven gut inflammation. Steroid therapy use in post-operative setting is not recommended for prophylaxis [50, 108]. Rutgeerts et al proposed an algorithm for the prophylaxis of Crohn's disease post-operatively as shown in figure 1.4.

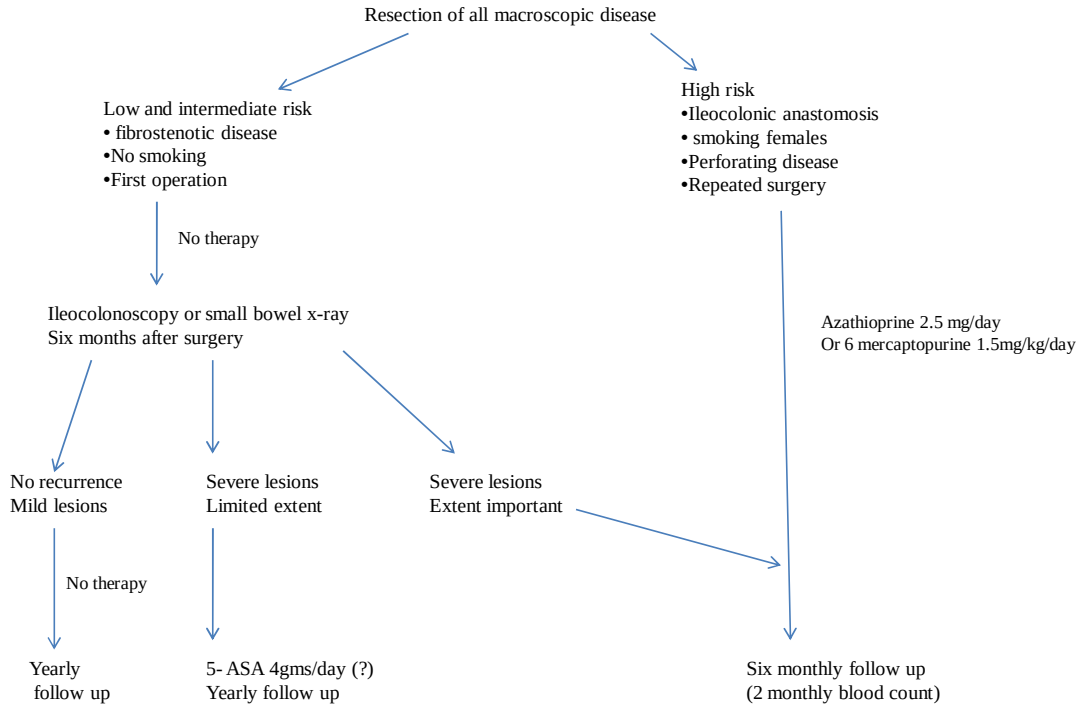

Figure 1.4 - Algorithm for prophylaxis of Crohn's recurrence after resection (Rutgeerts et al) [109].

1.9 Faecal Markers:

There is no gold standard test used in detecting and quantifying gastrointestinal inflammation. In about 70% of Crohn's disease patients, endoscopy and histology has been used to detect and quantify gastrointestinal inflammation. However this method is invasive, expensive, not well tolerated by patients and not examine small bowel completely [2]. In the search for less-invasive methods for diagnosis and monitoring of inflammatory bowel disease, faecal calprotectin and lactoferrin show

promise as simple, non-invasive, inexpensive sensitive and specific parameters to detect gastrointestinal inflammation [3-6]. Other various biological and serological markers such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Anti-saccharomyces cerevisiase (ASCA), anti-neutrophil cytoplasmic antibodies (ANCA), immunoglobulins IgA, Alpha-1-antitrypsin, Alpha-2-macroglobulin, faecal lysozyme, faecal myleperoxidase, faecal elastase, faecal lactate and whole gut lavage have low sensitivity and specificity for gastrointestinal inflammation and poorly correlate with disease activity [3, 7, 8, 110]. Although various markers have been investigated, none has been shown to be ideal or superior to our current diagnostic tools. Nevertheless, CRP is a useful marker and should be preferred in crohn's disease as it correlates well with disease activity in some studies but again have low sensitivity and specificity.

In conclusion, assessment of disease activity in patients with IBD is important both in clinical practice and in trials. The data is limited or inconsistent, however our knowledge of the inflammatory network involved in inflammatory bowel disease grows, and this may give rise to more sensitive and specific markers. As the main pathophysiological event in inflammatory bowel disease is neutrophils influx into the mucosa, neutrophils derived markers; especially faecal calprotectin and faecal lactoferrin show the most promising markers to assess disease activity in gastrointestinal inflammation.

1.9.1 Faecal Calprotectin:

Calprotectin was first described in 1980 by Fagerhol et al. It is a 36 kDA calcium and zinc binding protein and constitutes 60% of total soluble cytosol proteins in human neutrophil granulocytes. After granulocytes are released from bone marrow and reach gastrointestinal tract, they release antimicrobial substances which include calprotectin [111]. Hence calprotectin in faeces is directly proportional to leucocytes relocation to the gastrointestinal tract. A five day faecal excretion of leucocytes measured with Indium-111 has shown close correlation with faecal calprotectin in quantification of intestinal inflammation [112-115]. It is expensive, time consuming and exposes patient to radiation. Calprotectin has an antimicrobial function; it inhibits metalloproteinases and induces apoptosis in cell cultures [116, 117]. Once released from granulocytes its concentration increases over a hundred times the normal level in blood and faeces in rheumatoid arthritis and gastrointestinal inflammation [118-120]. It is stable for seven days at room temperature, which means that the faecal samples can be posted to a laboratory or brought directly to clinic. Five grams of faeces is enough to establish the presence of calprotectin using ELISA. Studies done in laboratories across England and in Norway have shown the normal level of faecal calprotectin in healthy individuals to be 50µg/g [2, 110, 118, 121, 122]. Individuals on non-steroidal anti-inflammatory medications which are associated with enteropathy have higher levels of faecal calprotectin. Also differences in diet and levels of

physical activity are associated with increased levels of faecal calprotectin [123-126] .

1.9.2 Faecal calprotectin as a marker for the diagnosis of inflammatory bowel disease:

The studies shown in the table 1.1 have examined the value of faecal calprotectin in diagnosis of inflammatory bowel disease in patients with symptoms suggestive of inflammatory bowel disease (both Ulcerative Colitis and Crohn's disease). The mean specificity and sensitivity for diagnosing inflammatory bowel disease is 76% (95% CI, 72-79%) and 80% (95% CI, 77-82%) respectively. Using faecal calprotectin as a marker for diagnosis, when compared between Crohn's disease and Ulcerative Colitis in above studies, a higher accuracy was seen in Crohn's disease (specificity 85% & sensitivity 83% in Crohn's disease, specificity 74% & sensitivity 72% in Ulcerative Colitis) [112, 127-138].

Using faecal calprotectin as a faecal biomarker against histological diagnosis of Inflammatory Bowel Disease a meta-analysis [139] of prospective studies was performed including healthy patients, patients with Crohn's disease, Ulcerative Colitis, and patients with Irritable Bowel Syndrome. Patients with Inflammatory Bowel Disease showed higher levels of faecal calprotectin (219.2 microgram/gram) compared to normal patients. Higher levels of faecal calprotectin were shown in Crohn's disease by 56microgram/gram when compared with Ulcerative Colitis [112, 127, 129,

133, 134, 140, 141].This test of faecal calprotectin didn't distinguish between Crohn's disease and Ulcerative Colitis clinically as the range of values used was very large.

A few studies conducted showed no significant difference in the levels of faecal calprotectin in distinguishing between Inflammatory Bowel Disease and Colorectal Cancer. The reason for this is that calprotectin is found elevated in many organic processes in the gastrointestinal tract such as Colorectal Cancer and gastrointestinal infections. In this situation where faecal calprotectin is elevated colonoscopy as follow up is required to rule out causes other than inflammatory bowel disease [139, 142]. In conclusion, studies have shown faecal calprotectin to be a better diagnostic faecal marker for inflammatory bowel disease than other markers such as C-reactive protein, erythrocyte sedimentation rate, ASCA and ANCA [112, 131, 139].

Table 1.1 - Diagnostic precision of faecal calprotectin for inflammatory bowel disease [131].

Study	Age	Patient & Disease	Cut-off level Microgram/gram	Sensitivity (%)	Specificity (%)
Carrocio et al [127]	Adults	10 CD	50	100	80
Carrocio et al [127]	Children	8 CD	50	100	96
Canani et al [128]	Children	17 CD 10 UC	100	93	89
Bunn et al [134]	Children	21 CD 16 UC	50	65	100
Bremner et al [133]	Children	43 IBD	50	95	72
D'Inca et al [130]	Adults	31 CD 46 UC	80	79	74
Thjodleifsson et al [136]	Adults	49 CD	50	88	91
Tibble et al [112]	Adults	116 CD	50	98	96
Fagerberg et al [140]	Children	22 IBD	50	95	93
Schroder et al [137]	Adults	25 CD 20 UC	15	93	100
Silberer et al [138]	Adults	39 IBD	18.6	61	-
Tibble et al [3]	Adults	31 CD	150	100	97
Limburg et al [132]	Adults	29 IBD	100	94	83
Kaiser et al [135]	Adults	32 CD 27 UC	50	63	86

1.9.3 Faecal calprotectin in the diagnosis of organic (small or large) gastrointestinal disease from functional disorder (IBS) in symptomatic patients:

Clinical symptoms of organic small or large intestinal (IBD or neoplastic disease) and functional disorder (IBS) range vary widely, so making specific diagnosis from symptoms alone difficult in symptomatic patients. Colonoscopy and biopsy is needed for accurate diagnosis. It is expensive, uncomfortable and needs general anaesthesia in paediatric patients. Using faecal calprotectin, studies have distinguished organic from functional gastrointestinal disease [112, 119, 127-129, 131] . These studies showed mean sensitivities of 83% (95% CI, 81-84%) and specificities of 84% (95% CI, 82-85%). In these studies, apart from faecal calprotectin, CRP and ESR were used for diagnostic accuracy of organic and functional disease [143-145]. Faecal calprotectin showed higher diagnostic accuracy than other two markers separately and in combination. In detection of colorectal cancer, faecal calprotectin was more sensitive than faecal occult blood [141]. In conclusion, from these studies faecal calprotectin could be used to distinguish between organic gastrointestinal (coeliac, diverticular, IBD and neoplastic diseases) from functional disease (IBS) in symptomatic patients and avoid unnecessary colonoscopies in the latter group [146].

1.9.4 Faecal calprotectin as a marker of disease activity and relapse in inflammatory bowel disease:

Several studies have been conducted comparing faecal calprotectin levels and disease activity based on clinical disease activity index, histological and endoscopic findings [112, 118, 119, 129, 134, 138, 147-152]. These studies found faecal calprotectin correlates more closely with histological findings than endoscopic appearance in evaluating inflammatory bowel disease activity [119]. The histological severity grades of colorectal inflammation were predicted with higher levels of faecal calprotectin [132]. Further studies looking at the disease extent were performed and these studies did not demonstrate the difference in levels of faecal calprotectin and disease extent of inflammation apart from disease activity [112, 129, 142]. Levels above 50 microgram/gram were better correlated with colitis disease activity index than Crohn's disease activity index (CDAI) [129, 130]. This might be due to Crohn's disease activity index being not sensitive enough to detect subclinical inflammation. In conclusion, further studies are needed to establish correlation between faecal calprotectin and disease activity index. A prospective study performed by Casellas et al demonstrated faecal calprotectin levels remained unchanged in clinically inactive Ulcerative Colitis and higher levels were seen in patient who relapsed when followed up for twelve months [152] .

Inflammatory bowel disease activity is unpredictable in its course of relapse and remission. Faecal calprotectin may have a role in predicting inflammatory bowel disease relapse. Higher levels of faecal calprotectin are

associated with increased rate of relapse. Study from from Tibble et al demonstrated that 90% of patients having high faecal calprotectin at the beginning of the study had relapsed within a year and only 10% of them had low levels of faecal calprotectin. Thus study concluded faecal calprotectin's sensitivity of 90% and specificity of 83% in predicting relapse [153]. Other serological markers like CRP and ESR did not demonstrate any usefulness in predicting relapse in patients who were in clinical remission. The difference in these markers could be explained that faecal calprotectin seems to be a direct marker of inflammatory activity while CRP and ESR estimate inflammation indirectly. Another study by Costa et al showed similar specificity (89%) and sensitivity (82%) in predicting relapse in patients with Ulcerative Colitis but lower specificity (43%) in patients with Crohn's disease. This study used a lower cut off (150microgram/gram equivalent to 30mg/litre) and different ELISA assay [154]. Also two other studies by Scarpa et al done on Crohn's disease, predicting relapse using faecal calprotectin levels did not show similar outcomes to Ulcerative Colitis. This difference could be due to different patterns of gastrointestinal inflammation in both diseases [155, 156]. In conclusion, more studies are needed on identifying this above difference between Crohn's disease and Ulcerative Colitis, and predicting relapse risk using faecal calprotectin.

1.9.5 Faecal calprotectin in the assessment of inflammatory bowel disease treatment response and after bowel resection surgery:

Only a few studies have been performed using faecal calprotectin as a biomarker in the assessment of disease activity clinically, endoscopically and histological improvement after treatment. These studies found the levels of faecal calprotectin decreased with treatment and correlated with clinical, endoscopic and histological progress in a single patient with Ulcerative Colitis over 18 week period [150]. Few others demonstrated a brief decrease in concentration with infliximab [157]. Follow up of fifteen patients on glucocorticoid therapy as treatment of active disease showed decrease in faecal calprotectin levels with clinical improvement but did not fall into the normal range, suggesting subclinical inflammation in clinically inactive disease. Discontinuation of treatment showed immediate increase in levels of faecal calprotectin [151]. This is in agreement with other studies showing that although patients with IBD achieved clinical remission with steroid therapy, microscopic remission is rarely achieved and persistent inflammatory changes were found in bowel in majority of patients.

Two studies have been performed so far to assess disease activity using faecal calprotectin on patients with Crohn's disease after bowel resection surgery. Orlando et al in 2006 consecutively followed 50 patients who had undergone previous resection for Crohn's disease had showed good correlation between faecal calprotectin of >200 mg can be an indication for colonoscopy and better sensitivity than ultrasound after 3 months of

resection surgery [158]. This study showed good correlation with endoscopic disease activity when faecal calprotectin levels were >200 mg but low sensitivity (75%) of faecal calprotectin and small sample size of 39 patients. Costa et al in 2003 showed that after bowel resection only patients with post-operative disease recurrence at the anastomotic site had increased levels of faecal calprotectin compared to normal levels in patients without disease recurrence [129]. In contrast, a study from Costa et al in 2007 showed increased levels of faecal calprotectin even after ileocolonic resection when followed up for long term [155]. This second study explained that even with good health, gastrointestinal inflammation persists and ileocolonic resection does not heal Crohn's disease. In summary, further prospective studies using faecal calprotectin as a biomarker are needed to identify its utility in assessment of inflammatory bowel disease medical treatment response and disease recurrence after bowel resection.

1.9.6 Faecal lactoferrin:

Faecal lactoferrin is a (76kDa) iron binding glycoprotein present in many body fluids such as human breast milk, synovial fluid, tears and in polymorphonuclear neutrophils. This protein is a major component of secondary granules in polymorphonuclear neutrophils and is secreted by mucous membranes [159]. This protein plays an important role in the defence mechanism (bacteriocidal properties of faecal lactoferrin) of mucous membranes during the inflammatory process as polymorphonuclear neutrophils degranulate during this process. Faecal lactoferrin concentration

in faeces is proportional to the influx of neutrophils translocation to the gastrointestinal tract on activation of polymorphonuclear neutrophils [160-162]. Faecal lactoferrin is stable at room temperature for a long period of time (seven days) and is suitable for reflecting inflammatory bowel disease activity. Like faecal calprotectin the quantity of faecal lactoferrin can be measured with simple enzyme linked immunosorbent assay (ELISA). Since faecal lactoferrin is stable at room temperature for seven days the samples can be posted to the laboratory or brought to the clinic [2, 138, 163, 164]. The normal level of faecal lactoferrin in healthy individuals measured by commercially available quantitative ELISA is less than 7.25 microgram/gram. Individuals currently on non-steroidal anti-inflammatory medications that are associated with enteropathy have higher levels of faecal lactoferrin [110, 122, 165].

1.9.7 Faecal lactoferrin as a marker for the diagnosis of inflammatory bowel disease:

Studies shown in the table 2.1 have demonstrated the diagnostic accuracy of faecal lactoferrin in diagnosis of inflammatory bowel disease in patients with clinical history of inflammatory bowel disease (both Ulcerative Colitis and Crohn's disease) [5, 6, 137, 163, 164, 166-172]. The mean specificity and sensitivity in diagnosing inflammatory bowel disease is 82% (95% CI, 79-84%) and 80% (95% CI, 78-83%) respectively. Using faecal lactoferrin as a marker for diagnosis, when compared between Crohn's disease and Ulcerative Colitis both had comparable specificity and sensitivity (77%

specificity & sensitivity 75% in Crohn's disease and in Ulcerative Colitis specificity of 74% & sensitivity 82%) [173]. When compared between faecal proteins like lysozyme, myeloperoxidase, faecal calprotectin and lactoferrin in the identification of gastrointestinal inflammation, until now information available is spare. Higher levels of faecal lactoferrin were shown in Ulcerative Colitis when compared with Crohn's disease possibly due to the short transit time of released neutrophils derived proteins [6, 148].The difference in levels of faecal lactoferrin between Ulcerative Colitis and Crohn's disease in these studies was suggested due to the heterogeneous nature of Crohn's disease and they were unable to adequately clarify to what degree the amount of ileal versus colonic disease was present in study patient. Studies have shown faecal lactoferrin to be a better diagnostic faecal marker for inflammatory bowel disease than other markers like C-reactive protein, erythrocyte sedimentation rate, ASCA, ANCA [144, 174, 175].

Table 2.1 - Diagnostic accuracy of faecal lactoferrin for diagnosis of Inflammatory Bowel Disease [173] .

Study	Control Group	Patient & Disease	Sensitivity (%)	Specificity (%)
Fine et al [169]	Different colonic diseases	103 IBD	90	98
D'Inca et al [130]	Different colonic diseases	77 IBD	76	67
Dai et al [171]	IBS and different colonic diseases	13 CD 42 UC	92 90	80 88
Hirata et al [170]	Different colonic diseases	40 CD 62 UC	63 47	-
Schroder et al [137]	IBS	45 IBD	82	100
Kane et al [5]	IBS	215 IBD	78	90
Saitoh et al [176]	Different colonic diseases	13 CD 18 UC	54 67	-
Walker et al [168]	IBS	106 IBD	87	68
Otten et al [177]	IBS	23 IBD 23 IBD	78 78	90 99
Langhorst et al [144]	IBS	43 CD 43 UC	82 90	60 67
Schoepfer et al [167]	IBS Other forms of colitis	36 IBD 36 IBD	86 100	100 14
Schoepfer et al [174]	IBS IBS	36 CD 28 UC	83 91	96 96

CD: Crohn's disease, UC: Ulcerative Colitis, IBS: irritable bowel syndrome.

1.9.8 Faecal lactoferrin in the diagnosis of organic (small or large) gastrointestinal disease from functional disorder (IBS) in symptomatic patients:

Similar to faecal calprotectin studies performed, faecal lactoferrin has distinguished organic (IBD or neoplastic disease) from functional (IBS) gastrointestinal disorders [112, 130, 178]. In contrast to faecal calprotectin, a study concluded in the detection of colorectal diseases (IBD, colorectal cancers and polyps), faecal lactoferrin and faecal occult blood tests were equally useful but cannot replace each other as screening tests [163, 179]. Faecal lactoferrin (as a marker of neutrophilic intestinal inflammation) could be used to distinguish between organic gastrointestinal diseases from functional disease in symptomatic patients and avoid unnecessary colonoscopies in the latter group [178].

1.9.9 Faecal lactoferrin as a marker of disease activity and relapse in inflammatory bowel disease:

Several studies have been conducted between faecal lactoferrin levels and disease activity based on clinical disease activity index, histological and endoscopic findings. These studies found faecal lactoferrin correlates closely with histological lesions in evaluating inflammatory bowel disease activity [6, 167, 168, 176, 180]. Faecal lactoferrin correlates significantly with histological lesions in Crohn's disease in contrast to faecal calprotectin. In Ulcerative Colitis and ileocolonic and colonic Crohn's disease faecal lactoferrin correlates significantly with endoscopic score [130, 172, 181]. A

study from Sipponen et al in 2008 on Crohn's disease demonstrated that in active disease increased levels of both faecal lactoferrin and faecal calprotectin were significantly higher in colonic than in ileal disease. Faecal lactoferrin, but not faecal calprotectin was significantly higher also in ileocolonic than in ileal disease. In strictly ileal disease, faecal lactoferrin correlated with endoscopic severity but not faecal calprotectin [172]. In another study close correlation between faecal lactoferrin levels and endoscopic and histological findings in patients diagnosed with Ulcerative Colitis was demonstrated. In patients with Crohn's disease this study only demonstrated correlation between faecal lactoferrin levels and histological findings but not with endoscopic appearances [130] . A study based on clinical disease activity in IBD has shown increased levels of faecal lactoferrin with a higher grade of disease activity. In the assessment of disease activity in inflammatory bowel disease both faecal lactoferrin and calprotectin are equally useful [155, 165].

Faecal lactoferrin was shown to predict inflammatory bowel disease relapse. Higher levels of faecal calprotectin are associated with increased rate of relapse. A study proved faecal lactoferrin's sensitivity is 62% and specificity is 65% in predicting relapse but when considering relapse in first three months a better sensitivity of 100% was obtained [182]. Another study in paediatric IBD patients showed higher levels of faecal lactoferrin in patients who relapsed clinically and normal levels in patients who were in clinical remission. This study also concluded that persistent higher faecal lactoferrin

levels may predict early relapse in patients tapering off steroid therapy [168]. In conclusion, more studies are needed in predicting relapse risk after bowel resection surgery, and order of relapse using faecal lactoferrin.

1.9.10 Faecal lactoferrin in the assessment of inflammatory bowel disease treatment response and after bowel resection surgery:

Only two studies have been performed using faecal lactoferrin as a biomarker in the assessment of disease activity clinically, endoscopically and histological improvement after medical treatment. These studies found levels of faecal lactoferrin decreased rapidly after first infusion of infliximab and also in all patients who responded clinically after the first infusion [183, 184].

Two studies have been performed so far to assess disease activity using faecal lactoferrin on patients with Crohn's disease after bowel resection surgery [155, 185]. Firstly, Scarpa et al showed that faecal lactoferrin levels stayed high after surgery even in patients who were in remission. They also observed that faecal lactoferrin levels were significantly increased in patients who had clinical recurrence. Secondly, a study from Parsi et al done on IBD patients with an ileoanal pouch showed faecal lactoferrin levels to be high in pouchitis with normal levels in symptoms from irritable pouch syndrome. This study also concluded that faecal lactoferrin as a initial diagnostic test in symptomatic pouch patients to be cost effective when compared with metronidazole therapy alone and pouch endoscopy with biopsy.

1.10 Aims of study:

Previous research has shown both faecal calprotectin and lactoferrin are able to differentiate organic from functional disease, simple to use, reliable, inexpensive and sensitive for gastrointestinal inflammation and non-invasive. The clinical usefulness of faecal calprotectin and lactoferrin in the post-operative setting has not been prospectively established.

Quantitative measurement of faecal calprotectin and faecal lactoferrin may offer a valuable method as non-invasive markers in assessing the symptomatic post-operative patient in Crohn's disease.

To investigate this above hypothesis this study had the following aims:

1) To determine whether faecal calprotectin and faecal lactoferrin measurements in symptomatic post-operative Crohn's patient can differentiate those with early disease relapse from those with late relapse.
2) To compare faecal calprotectin and faecal lactoferrin with other measurements of inflammatory activity such as C-reactive protein, white cell count, platelet count, clinical disease activity index (HBI) and endoscopic finding.
3) To identify the heterogeneous group of patients (those with mild to moderate disease activity) using CRP, white cell count and faecal calprotectin and lactoferrin and there correlation and also looking at smoking status and maintenance therapy with these markers.

4) To determine how faecal calprotectin and faecal lactoferrin correlate with each other after resection.

5) To determine the immediate post-operative course of faecal calprotectin and faecal lactoferrin after ileocaecal resection.

6) To determine whether these faecal markers could demonstrate inflammatory activity and identify post-operative disease recurrence.

7) To identify if there is a difference in this two markers among uncomplicated and complicated group (with post-operative complications like anastomotic leak, intra-abdominal collection, wound infection and disease recurrence) of patients in immediate post-operative setting.

This study involved investigation of two related arms: cross sectional and longitudinal studies. The Cross sectional study addresses the first four aims of the study and findings are shown in chapter-3 of this thesis. The longitudinal study addresses the last three aims of the study and findings are shown in chapter-4 of this thesis. This study was designed to be achievable in the time frame and within the budget available as well as acceptable to patients by virtue of not involving any invasive tests.

Chapter 2

Materials and Methods

2.1 Brief Overview:

In this section I have outlined my personal contributions to the study and the areas where I have been helped by others to complete this project. I began my work by writing a rough study design protocol and presented it to my supervisor in November 2005. After discussing it with my supervisor we came to an agreement for a final study. Subsequently I started to apply for funds for the project. I will discuss the study design details later in this section of the thesis. I applied to a surgical research fellowship at Royal College of Surgeons, London. I was shortlisted for funding there but did not get it. Through my supervisor I got introduced to (IBD-SCAN®; Techlab, Blacksburg, Virginia, USA) ScheBo Biotech Company (Basingstoke, UK supplier). I discussed my project with the director of ScheBo Company. I got approved for free faecal lactoferrin kits for the project. The remaining funding was provided by my supervisor Dr. J.C. Mansfield's' research funds. I was employed as a research fellow at Newcastle upon Tyne Hospitals NHS Trust who covered the rest of the funding for my on-call clinical commitments. I was employed fulltime as clinical research fellow with on-call commitments from October 2005 until December 2007.

I applied to the Research and Development department at Newcastle upon Tyne NHS Trust to act as a sponsor for this project under the department of health guidelines for research in health and social care and got approval from them. Next I applied to Gateshead and South Tyneside Ethics Committee (Reference No. 06/Q0901/4). I was called for an interview along

with my supervisor by the Ethics Committee. The project was explained to them. I was advised by them to make a few corrections on my application form (patient information leaflets and consent forms). After doing that the committee accepted my application and granted ethical approval for this study in March 2006.

I began by attending IBD clinics in Newcastle to recruit patients. I approached patients who had ileocaecal resection for Crohn's disease (for cross-sectional study) and also patients who were planned for undergoing ileocaecal resection (for longitudinal study). I explained the project to the patients. Once they agreed to participate in the project I would take consent and obtain clinical data from them. I would explain to them that they need to give a faecal sample. For this they would be provided a faecal sample collection kit (figure 2.1) and postal kit (figure 2.1a) which they take home. They get a faecal sample at home and post it to the laboratory. For patients who did not have facility to post the faecal sample I made arrangements to personally go to their home and pick up the samples.

The faecal sample kit consisted of a faecal collection bag, test tube with spatula, test tube holder, cardboard box, and self addressed stamped envelope. During the interview I explained to the patient how to collect a faecal sample in the faecal bag. The patient was asked to collect a faecal sample in the faecal collection bag, take a small amount from the bag with the spatula, put it in the test tube and seal it. The test tube with faecal

sample was to be put in the test tube holder, then in the cardboard box, which was to be put into the envelope and posted to the laboratory. I advised them to post the sample within 24 hours of collection. Once I received the sample in the lab I would freeze it at -20°c. I would freeze all the samples until I had enough samples to run the ELISA.

Before beginning my ELISA experiments I went to learn this technique at the Department of Biochemistry at Guy's, King's, St.Thomas Medical School, London under Dr. Roy Sherwood, where they were running the faecal calprotectin and lactoferrin ELISA routinely. After learning this technique in London my supervisor and I approached Dr.Dermat Neeley (Head of Department of Biochemistry, Newcastle upon Tyne Hospitals) to set up a facility for me to run this assay in Newcastle. Under the supervision of biochemist Mrs.J.Gicquel, I started to run the assay initially on healthy volunteers to master both techniques (FC and FL). Once I perfected the technique I began to do the experiments on actual patient samples independently. The techniques of the experiments are discussed in details in the following paragraphs and results of the experiments are discussed in detail in the results section of the thesis.

The findings of this research project have been presented and published by myself in many medical and surgical meetings. I have discussed this in more detail in the publication section of this thesis.

2.2 Study design:

This study involved investigation of two related arms: cross sectional and longitudinal studies. The Cross sectional study was used to determine whether faecal calprotectin and lactoferrin concentration measurement could identify gut inflammation in patients with symptomatic Crohn's disease in the postoperative period and longitudinal study to monitor the trend of faecal calprotectin and lactoferrin concentrations in the immediate postoperative period of Crohn's disease patients. This study was designed to be achievable in the time frame and within the budget available as well as acceptable to patients by virtue of not involving any invasive tests.

2.3 Patient Selection:

All patients included in both arms had a histological diagnosis of Crohn's disease prior to inclusion and had an ileocaecal resection between January 1982 and August 2007. All patients were recruited from medical and surgical gastroenterology clinics at Newcastle upon Tyne Hospitals NHS Foundation Trust. 140 patients in total were included in the study but 23 patients were lost at the stage of faecal sample collection so a total of 117 patients were studied. The demographics of the patients included are shown in table 3.1 in the results section of this thesis. The normal upper limit ranges for the faecal calprotectin (< 50 µg/g) and lactoferrin (< 7.25 µg/g) were well defined so a control group was not required in this study. The Gateshead and South Tyneside local research and ethics committee approval was obtained for the study reference number 06/Q0901/4. All

patients included in the study gave a written informed consent before inclusion in the study. An example of the patient information sheet and consent form is in the appendix of this thesis.

2.3.1 Cross-sectional Study:

A total of 104 patients with a mean duration of 24 (range 2-300) months since surgery were recruited. All patients had undergone a previous ileocaecal resection for Crohn's disease. The following clinical characteristics were recorded from patient's notes and direct interview: age at disease or symptom onset, smoking history, sex, disease location, disease behaviour, pharmacology therapy and ileocolonoscopy findings (considered valid if performed within 4 weeks of patient interview). To estimate clinical disease activity Harvey Bradshaw Index Score [186] was also calculated for each patient. A Score <3 indicated clinically inactive, 4-mildly active, 5-moderately active and >6 severely active disease. Venous blood was obtained for white cell count (normal range 4.3-10×10^9/L), C-reactive protein (< 5miligram/L) and platelets (normal range 150-350×10^9/L). On the same day a single stool sample for faecal calprotectin and lactoferrin was collected.

2.3.2 Longitudinal Study:

A total of 13 patients who had undergone ileocaecal resection were followed for 12 months for symptomatic Crohn's disease. The following clinical characteristics were recorded from patient's notes and direct interview: age

at disease or symptom onset, smoking history, sex, disease location, disease behaviour, current pharmacology therapy and ileocolonoscopy findings (considered valid if performed within 4 weeks of patient interview). Data on the complications, need for further interventions, postoperative course and outcome were collected. Serial stool samples including before surgery, at weekly intervals for 4 weeks and monthly intervals for 12 months were collected to determine the trends in faecal calprotectin and lactoferrin levels after surgery.

2.4 Faecal sample collection and storage:

Patients were taught to collect the faecal sample and a faecal sample collection bag along with the laboratory addressed postal kit (figure 2.1 & 2.1a) was given to patients by me during their interview. For faecal calprotectin and lactoferrin levels, a single stool sample was used. The samples were stored at -20℃ after collecting, labe lling until assays was performed. The assays were performed in the clinical biochemistry laboratory at Freeman Hospital, Newcastle upon Tyne NHS foundation Trust. Ten samples were obtained from healthy control patients without inflammatory bowel disease to confirm the quoted normal ranges for the two stool markers.

Faecal calprotectin was measured using a commercially available (PhiCalTM; Calpro, Lysaker, Norway) quantitative enzyme immunoassay kit. Faecal lactoferrin was measured by (IBD-SCAN®; Techlab, Blacksburg, Virginia, USA) quantitative enzyme-linked immunosorbent assay. The

manufacturer's guidelines were followed for the technique and measurements of both faecal calprotectin and faecal lactoferrin. These techniques have been successfully used in previous research projects [5, 155, 183]. Venous blood was obtained for white cell count (normal range 4.3-10 $\times 10^9$/L), C-reactive protein (< 5miligram/L) and platelets (normal range 150-350 $\times 10^9$/L).

Figure 2.1 - Faecal sample collection kit.

Figure 2.1a - Faecal sample postal kit.

2.5 Harvey-Bradshaw Activity Index:

As most symptoms of Crohn's disease broadly affect quality of life, attempts have been made to incorporate physical, social and emotional performance characteristics into tests for assessing severity of Crohn's disease. While Crohn's Disease Activity Index is considered closest to being gold standard but not been validated [187, 188]. A key criticism is that Crohn's disease activity index does not incorporate a subjective assessment of quality of life, endoscopic factors or systemic features into calculation. Also it involves assessment over a period of seven days. Hence in this study much simpler version of the Crohn's Disease Activity Index, Harvey Bradshaw Activity

Index was used. The Harvey Bradshaw Activity Index is a research tool used for quantifying the symptoms of patients with Crohn's disease table 2.2. The score ranges from 0 to 25, a score <3 indicated clinically inactive, 4- mildly active, 5-moderately active and >6 severely active disease. It includes number of liquid stools, abdominal pain (0=none, 1=mild, 2=moderate, 3=severe), general well being (0=very well, 1=slightly below par, 2=poor, 3=very poor, 4=terrible), abdominal mass (0=none, 1=dubious, 2=definite, 3=definite and tender) and complications (arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure- score 1 per item) [186]. It is simpler than the CDAI score [189] which requires a 7 day symptom diary.

Table 2.2 - Harvey Bradshaw Activity Index (HBI)

General Well-being	Very well=0 Slightly below par=1 Poor=2 Very poor=3 Terrible=4
Abdominal pain	None=0 Mild=1 Moderate=2 Severe=3
Number of liquid stools per day	
Abdominal mass	None=0 Dubious=1 Definite=2 Definite and tender=3

Complications	Arthralgia=1 Uveitis=1 Erythema nodusum=1 Apthous ulcers=1 Pyoderma gangrenosum=1 Anal fissure=1 New fistula=1 Abscess=1

2.6 Statistical Analysis:

Power calculation and sample size for the study was confirmed after consultation with Newcastle University statistician (Prof Mathews). The minimum primary analysis for the cross-sectional study was to determine whether faecal measurements (of calprotectin and lactoferrin) were more likely to be elevated in active post-operative disease. In a population with 40% inactive disease a sample size of 50 would be sufficient to detect a statistically significant association if the test is 80% effective at discriminating active disease from inactive disease. In this study over 100 patients were available and the larger sample size gave the possibility to do much more detailed statistical testing as presented in the results section. The longitudinal study is essentially a case series to illustrate the value of sequential serial data, it is inappropriate for a sample size calculation and as it is limited by the availability of resections done during the study.

Sample size of one hundred patients in the cross-sectional study was sufficient to identify any clinically useful difference between faecal calprotectin and faecal lactoferrin. It was also sufficient to identify any clinically useful difference in faecal calprotectin and faecal lactoferrin concentrations between those with aggressive recurrent disease and those with indolent or inactive disease.

The longitudinal study was a descriptive study looking at the time course of the faecal calprotectin and faecal lactoferrin changes in the post-operative time period. The sample size was ten patients as this reflects the selection of patients who will undergo surgery prospectively.

SPSS® for Windows® version 15.0 (SPSS, Chicago, Illinois, USA) was used for data analysis. Kruskal-Wallis test was used for non-parametric data to identify significant differences in groups of continuous variables. To compare faecal calprotectin or faecal lactoferrin and continuous parameter, the Spearman's rank order correlation (r) was used.

2.7 Method- faecal calprotectin experiment:

The PhiCal^{TM}; Calpro, Lysaker, Norway test is a commercially available kit, used as quantitative ELISA for measuring concentration of faecal calprotectin (figure 2.2) in this study.

2.7.1 Principle of test:

Antibodies to rabbit calprotectin are used in this ELISA. Once the faecal specimen extract is obtained and centrifuged, the sample is used for ELISA for faecal calprotectin. The rabbit antibodies react with at least 6 different epitopes on calprotectin which will ensure a positive signal even if some of the epitopes are destroyed by other substances in the stool. Hence a use of monoclonal antibodies involves a risk of giving false negative results and is not used in this assay. The PhiCal^{TM} kit used for the test has microassay wells that contain polyclonal antibodies against calprotectin. Standards and serial dilutions of faecal specimens are put in the microassay wells. Later incubated for 45 minutes the wells are washed and antibody conjugate is added. During incubation period the conjugate binds with immunoaffinity purified anti-calprotectin. Again the wells are washed to remove any unbound material and substrate is added. If calprotectin is present the enzyme antigen-antibody complex will demonstrate a colour after a second incubation period of 20-30 minutes. Hence, the amount of enzyme bound is proportional to the amount of faecal calprotectin in the sample or standard, which can be determined after incubation with substrate for the enzyme. The test is run on stool extracts prepared by the use of patented extraction

buffer present in the kit. This buffer brings calprotectin present in stool into solution and has a molecular configuration similar to that of leukocytes extract or plasma. The reason for having similar configuration is important for this quantitative immunoassay. This assay requires both proteins in the standards and samples to have the same configuration. The standard curve is generated for calprotectin, sample values < 50 µg/g are considered negative and values of ≥ 50µg/g are considered to be elevated.

2.7.2 Contents of the kit and preparation of reagents:

a) Extraction solution- the solution provided in the kit contains 2X 90ml of the 2.5X concentrate. To obtain 225ml of extraction solution 135 ml of distilled water is added and mixed well to use for the test.

b) Washing solution- the solution provided in the kit contains 2X 50ml of the 20X concentrate. To obtain 1000ml of the washing solution 950ml of distilled water is added.

c) Sample diluents solution- the solution contains 20ml of 10X concentrate. To obtain 200ml of diluents solution 180ml of distilled water is added.

d) Enzyme conjugate antibody- the solution contains 16ml of alkaline phosphatise labelled immunoaffinity purified IgG antibodies from rabbit against calprotectin. It is a protein buffered solution containing sodium azide as a preservative.

e) Enzyme substrate solution- the solution contains ready to use 16ml of substrate in buffer.

f) Standards- the solution contains 1.2ml of known concentrations of calprotectin. Total of 8 vials are provided with different concentrations (Standard A-7.8ng/ml, Standard B-15.6ng/ml, Standard C-31.3ng/ml, Standard D-62.5ng/ml, Standard E-125ng/ml, Standard F-250ng/ml, Standard G-500ng/ml and Standard H-1000ng/ml) as shown in the figure below.

g) Control Solution- the solution 1.2ml of human calprotectin. The range of value is printed on the vial label and expressed in ng/ml.

h) Stop solution- the solution contains 1M of NaOH.

i) Microassay plate- it contains 12 strips and 8 wells in each strip. Each well is coated with polyclonal rabbit antibodies specific for calprotectin. The plate in a sealed bag with desiccant.

Figure 2.2 - PhiCal™ kit; Calpro, Lysaker, Norway.

2.7.3 Faecal sample collection and preparation:

Faecal samples of 1-5g received by post were stored at -20°C, if the test not performed within 48hrs. Approximately 100mg of faecal sample taken out, weighed with inoculation loop and prediluted extraction buffer is added to a weight/volume ratio 1:50 and mixed vigorously for 30 seconds. The sample along with extraction buffer is mixed on a shaker at approximately 1000 rpm for 30 minutes (figure 2.3). Then 1-2ml is transferred to a tube and centrifuged at 10000g for 20 minutes at +4°C for 20 minutes. The supernatant is the extract used to dilute and run for ELISA or could be stored at +4°C for several days or for 12 months if frozen. Therefore the extract is diluted in a ratio of 1:50 (20 µl faecal sample + 980 µl dilution buffer) before running the ELISA. Faecal samples with high concentrations are needed for re-testing after further dilution (1:5).

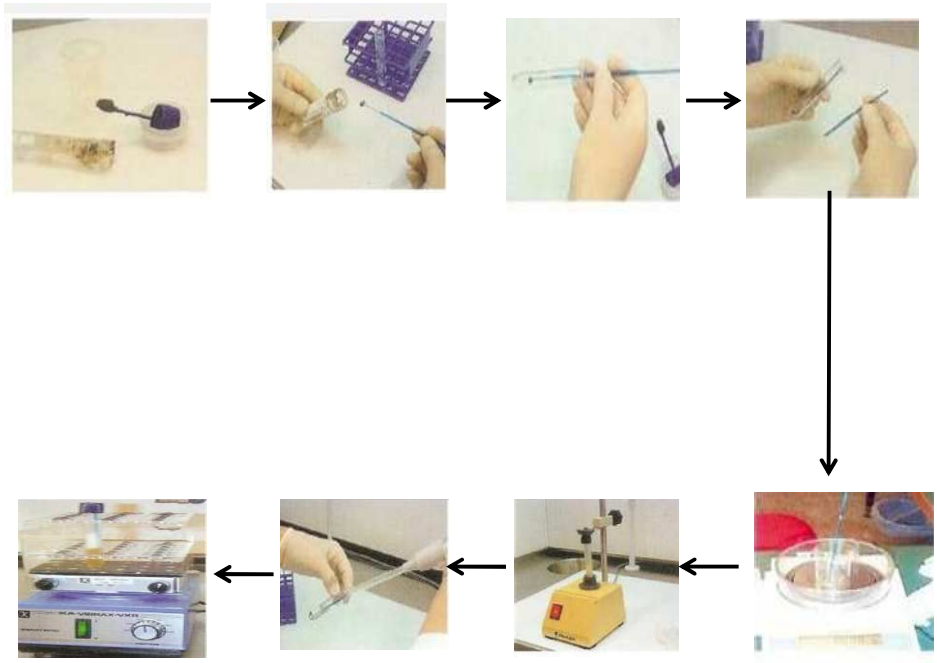

Figure 2.3 - Sample collection and preparation (PhiCal^{TM} kit; Calpro, Lysaker, Norway).

2.7.4 Assay procedure:

Faecal samples are collected, prepared and thawed at room temperature. 50 µl of diluted faecal sample, standard and control was added to wells as shown in the layout below (figure 2.4) and incubated at room temperature on a horizontal shaker for 45±5 mins. Later wells are washed with 250 µl washing buffer and this step is repeated for 5 washings. The plate is inverted and tapped on an absorbent tissue to remove washing buffer. After adding 50 µl of conjugate to each well, the plate is incubated at room temperature for 45±5 mins. Once the incubation period is completed the washing of the wells is repeated for 5 times again with the washing buffer.

100 µl of substrate is added using multi-channel pipette. The final incubation is carried out for 30 minutes in the dark until the optical density reading of the 1000ng/ml standard is 2.0 or stop solution is added to each well. The optical densities of all the wells are read on ELISA reader at 405 nm.

	1	2	3	4	5	6
A	STANDARD H 1000ng/ml	STANDARD H 1000ng/ml	CONTROL	CONTROL	SPECIMEN 8	SPECIMEN 8
B	STANDARD G 500ng/ml	STANDARD G 500ng/ml	SPECIMEN 1	SPECIMEN 1	SPECIMEN 9	SPECIMEN 9
C	STANDARD F 250ng/ml	STANDARD F 250ng/ml	SPECIMEN 2	SPECIMEN 2	SPECIMEN 10	SPECIMEN 10
D	STANDARD E 125ng/ml	STANDARD E 125ng/ml	SPECIMEN 3	SPECIMEN 3	SPECIMEN 11	SPECIMEN 11
E	STANDARD D 62.5ng/ml	STANDARD D 62.5ng/ml	SPECIMEN 4	SPECIMEN 4	SPECIMEN 12	SPECIMEN 12
F	STANDARD C 31.3ng/ml	STANDARD C 31.3ng/ml	SPECIMEN 5	SPECIMEN 5	SPECIMEN 13	SPECIMEN 13
G	STANDARD B 15.6 ng/ml	STANDARD B 15.6ng/ml	SPECIMEN 6	SPECIMEN 6	SPECIMEN 14	SPECIMEN 14
H	STANDARD A 7.8ng/ml	STANDARD A 7.8ng/ml	SPECIMEN 7	SPECIMEN 7	SPECIMEN 15	SPECIMEN 15

Figure 2.4 - Plate template layout of test samples- including calprotectin standards, control and faecal specimens (PhiCal^{TM} kit; Calpro, Lysaker, Norway).

2.7.5 Calculation of faecal calprotectin results:

The concentration of faecal calprotectin in all the wells is calculated by a computer linked ELISA reader (4-parameter or spline function) or manually. The mean optical densities of all the duplicate wells are calculated. To obtain a standard curve a graph is plotted using log values of the standards against their own optical densities (as shown in figure below), control value readings should be within the value printed on the bottle (as shown in the figure 2.5). The values of the faecal samples from the standard curve are corrected for dilution and converted to mg/kg by multiplying by 2.5 and the additional dilution factor is entered if faecal samples are further diluted during calculation. Faecal samples giving readings above 50µg/g are regarded as positive for calprotectin

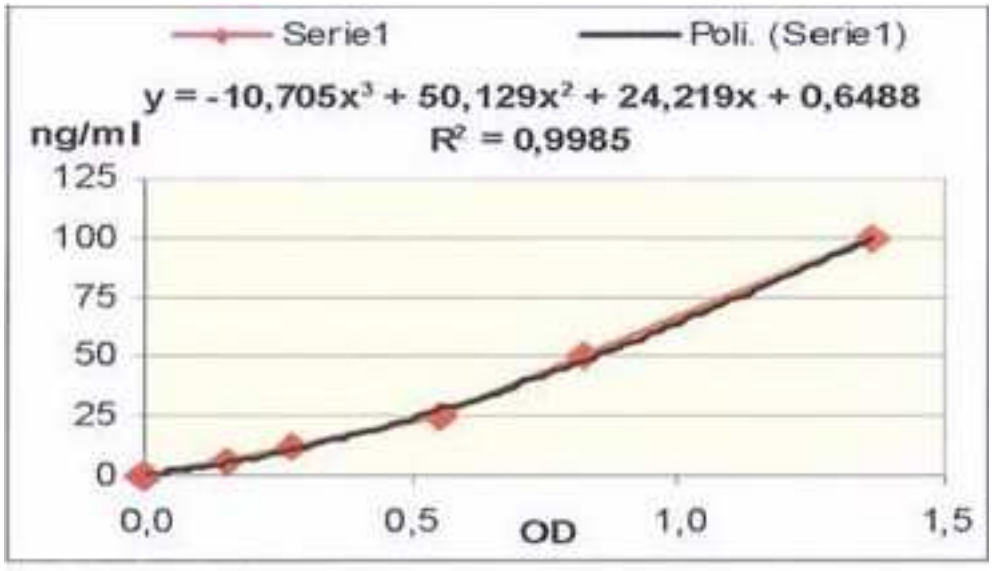

Figure 2.5 - Calprotectin standard curve for optical density of 405 nm (PhiCal^{TM} kit; Calpro, Lysaker, Norway).

2.8 Method- Faecal lactoferrin experiment:

The ELISA IBD-SCAN® (Techlab, Blacksburg,Virginia,USA) test is a commercially available quantitative test for measuring concentrations of faecal lactoferrin which is a marker of faecal leukocytes [161] (figure 2.6) used in this study.

2.8.1 Principles of test:

Antibodies to human lactoferrin are used in this test. The IBD-SCAN®; Techlab kit used for the test has microassay wells that contain immobilised polyclonal antibody against lactoferrin. The detecting antibody is made of polyclonal antibody conjugated to horseradish peroxidise. Standards and serial dilutions of the faecal specimens are put in the microassay wells. The faecal lactoferrin binds to immobilised antibody if there are measurable levels of lactoferrin in the faecal specimen. After 30 minutes of incubation the wells are washed and antibody conjugate is added. During this incubation period the conjugate binds to the lactoferrin. Again the wells are washed to remove any unbound material and substrate is added. If lactoferrin is present the enzyme antigen-antibody complex will demonstrate a colour after a second incubation period of 15 minutes. The concentration of lactoferrin present is directly proportional to absorbance measured. The standard curve generated for lactoferrin ranges from 6.25 to 100 ng/ml. Lactoferrin concentration can be determined in a test sample by plotting absorbance values against lactoferrin concentrations.

2.8.2 Contents of the kit and preparation of reagents:

a) Diluent- the solution contains 10X concentrate of a buffered protein solution containing 0.2% thimersol.40 ml of diluents is supplied as a 10X concentrate in the kit. 360ml of deionised water is added to 40 ml provided to dilute to obtain a total volume of 400ml. The 1X diluent is used as negative control.

b) Wash buffer solution- the solution contains 20X concentrate of phosphate buffered saline, detergent and 0.2% thimersol. 50ml is supplied as a 20X concentrate in the kit. 950 ml of deionised water is added to the 50 ml provided to obtain a total volume of 1 litre.

c) Conjugate- the solution contains 7 ml of rabbit polyclonal antibody specific for human lactoferrin conjugated to horseradish peroxidise. It is in a buffered protein solution containing 0.02% thimerosal.

d) Substrate- the solution contains 14ml of tetramethylbenzidine substrate and peroxide.

e) Standards- the solution contains 1.5 ml of human lactoferrin standard. It is in a buffered protein solution contacting 0.02% thimerosal. Total of 5 standard solutions are provided with different concentration (LS1-100ng/ml, LS2-50ng/ml, LS3-25ng/ml, LS4-12.5ng/ml and LS5-6.25ng/ml) as shown in figure below.

f) Positive control- the solution contains 1.5ml (10µg/ml) of human lactoferrin. It is in protein buffered solution containing 0.02% thimerosal. 1:10 and 1:200 dilution of positive control is prepared in two tubes. Two tubes containing 450 µl & 950 µl of 1X diluents are taken. 50 µl of positive

control is added to tube 1 and 50 µl from tube 1 to tube 2. Both the tubes are vortex for 10 seconds and stored between 2 & 8°C till ELISA performed. Therefore tube 1 contains 1:10 and tube 2 contains 1:200 dilution of positive control.

g) Stop solution- the solution contains 7ml of sulphuric acid.

h) Microassay plate- it contains 12strips and 8 wells in each strip. Each well is coated with purified polyclonal antibody for lactoferrin.

Figure 2.6 - IBD-SCAN® kit; Techlab, Blacksburg, Virginia, USA.

2.8.3 - Faecal sample collection and preparation:

Once a faecal sample is received by post it is stored at -20°C, if the test is not performed within 48 hrs. The diluted faecal sample is prepared by setting up 3 tubes for 1:10 and 1:200 concentrations. 450µl of diluents is added to three tubes and 50µl or 0.05g of faecal specimen is added to tube one and mixed. Then 50µl is transferred from tube 1 to tube 2 and mixed well and finally 50µl is transferred from tube 2 to tube 3 mixed well on a vortex mixer. Therefore tube 1 contains 1:10, tube 2 contains 1:100 and tube 3 contains 1:1000 diluted faecal samples for the assay.

2.8.4 Test procedure:

Materials provided in the kit are shown in the figure 2.6 also contains 2 plastic adhesive sheets and 100 transfer pipettes. Other materials required for the experiment are a refrigerator for storage, discard container/absorbent paper, distilled water, squirt bottle for wash buffer solution, vortex mixer, tubes for dilution of specimen, bottle for diluents, pipettes and ELISA reader (450 nm or 450/620 nm). Following the completion of preparing diluent, wash buffer, positive control, and negative control and diluted faecal specimen the ELISA using the microassay plate is started. Using designated and two wells for each standard, one well for 1:200 dilution of positive control, one well for negative control (1X diluent) and one well for faecal specimen dilutions 1:100 and 1:1000 as shown in the figure 2.7.

	1	2	3	4	5	6
A	NEGATIVE CONTROL	POSITIVE CONTROL(100)	SPECIMEN3 (100)	SPECIMEN3 (1000)	SPECIMEN11 (100)	SPECIMEN11 (1000)
B	STANDARD 1	STANDARD1	SPECIMEN4 (100)	SPECIMEN4 (1000)	SPECIMEN12 (100)	SPECIMEN12 (1000)
C	STANDARD 2	STANDARD2	SPECIMEN5 (100)	SPECIMEN5 (1000)	SPECIMEN13 (100)	SPECIMEN13 (1000)
D	STANDARD 3	STANDARD3	SPECIMEN6 (100)	SPECIMEN6 (1000)	SPECIMEN14 (100)	SPECIMEN14 (1000)
E	STANDARD 4	STANDARD4	SPECIMEN7 (100)	SPECIMEN7 (1000)	SPECIMEN15 (100)	SPECIMEN15 (1000)
F	STANDARD 5	STANDARD5	SPECIMEN8 (100)	SPECIMEN8 (1000)	SPECIMEN16 (100)	SPECIMEN16 (1000)
G	SPECIMEN 1 (100)	SPECIMEN1 (1000)	SPECIMEN9 (100)	SPECIMEN9 (1000)	SPECIMEN17 (100)	SPECIMEN17 (1000)
H	SPECIMEN 2 (100)	SPECIMEN2 (1000)	SPECIMEN10 (100)	SPECIMEN10 (1000)	SPECIMEN18 (100)	SPECIMEN18 (1000)

Figure 2.7 - Plate template layout of test samples- including lactoferrin standards, positive and negative control and faecal specimens (IBD-SCAN®; Techlab, Blacksburg, Virginia, USA).

100µl of each standard (LS1-LS5) is added using a calibrated pipette to duplicate wells and 100µl of negative control (1X diluents) and positive control (1:100) is added to designate wells as shown in the table 2.7 above. Also 100µl of faecal specimen diluted is added using a fresh pipette to separate wells (1:100 & 1:1000 dilution). The wells are covered with adhesive plastic sheet and incubated at 37℃ ±2℃ f or 30 minutes.

Once the incubation period is completed for 30 minutes the contents of the assay wells are discarded and each well is washed for five times using 1X wash solution using the squirt bottle. The direction of wash solution should be towards the bottom of the well with force and the plate is inverted on dry absorbent paper and slapped each time of wash. The washing should be continued if any particulate matter is seen in the wells until it is cleared from the well. One drop of conjugate is added provided in the red cap for all the wells and further incubated for 30 minutes at 37℃ ±2℃. Later, after incubation the washing of wells is repeated as before for five times and dried on absorbent paper.

Two drops of substrate are added into each of the wells and incubated for fifteen minutes at room temperature. Gentle tapping of wells is done one to two times to mix the contents during the incubation period. Finally one drop of stop solution is added and rested for 2 minutes before reading of plate is done. This addition of stop solution converts the blue colour of the solution to yellow and the optical density is then quantitatively measured at 450 nm or 450/620 nm on ELISA plate reader. The reading of plate is done within 2 to 10 minutes of adding stop solution. All the absorbance values are recorded for the standards, positive and negative control and each faecal specimen. Lastly, before interpreting of the results, the average value is taken of the duplicate wells. The quality control of the assay is assessed and should have values of negative control optical density of <0.100 for 450 nm and <0.060 for 450/620 nm and positive control value of 10±3 µg/ml.

The R^2 value should be ≥ 0.98 when the linear trend/regression type analysis is used and if the graph is followed then the points should be in a straight line as shown figure 2.8.

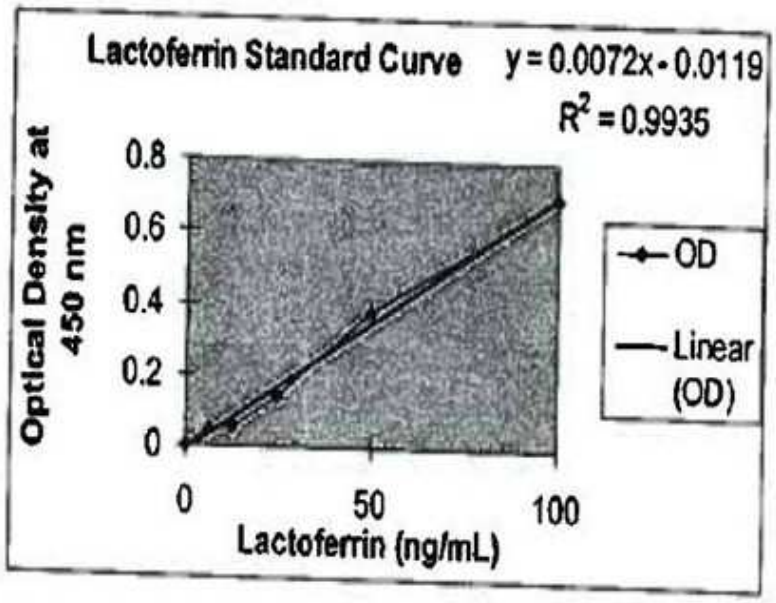

Figure 2.8 - Lactoferrin standard curve for optical density of 450 nm (IBD-SCAN®; Techlab, Blacksburg, Virginia, USA).

2.8.5 Calculation of faecal lactoferrin results:

Using linear trend/regression type analysis on an appropriate data reduction computer programme optimal estimation of faecal specimen values is obtained. The most diluted specimen giving values within the standard curve if optical density 450 nm ≥ 0.100 and optical density 456/620 nm ≥ 0.060 is chosen. The test is repeated using dilution of 1:10 if both the sample dilutions have values greater than the highest concentration of standard. Any specimen value showing less than the lowest concentration should also be retested using dilution of 1:10. If the result is found to be

negative then it's recorded as < 1µg/g. The average values obtained for standards are plotted on y-axis and the concentrations are plotted on x-axis to perform the linear/regression analysis. The R^2 value obtained should be ≥ 0.98.

The programme to produce the equation (Y=MX+B) for the plotted line is inserted in the computer, where X equals concentration of unknown specimen, M equals slope, B equals Y-intercept and Y equals optical density 450 nm or 450/620 nm of the specimen. Later X is determined as the concentration of the faecal lactoferrin in the specimen and multiplied by dilution factor (which could be 10 for 1:10 dilution, 100 for 1:100 and 1000 for 1:1000 dilutions) and divided by 1000 to convert ng/ml to µg/ml. The value obtained for lactoferrin in the faecal specimen is interpreted to be normal if it is between 0-7.24 µg/ml and elevated if ≥ 7.25 µg/ml.

The limitations of the IBD-SCAN® are as follows: it could only be used in faecal specimen as other types of clinical specimens are not evaluated, not to be used in immuno-compromised patients or with patients with history of ileostomy formation within 1 month or history of infectious diarrhoea or faecal specimen preserved in formalin or patients with colorectal cancer as this gives high values.

Chapter 3

Cross-Sectional Study

Results

3.1 Introduction:

To determine whether faecal calprotectin and faecal lactoferrin concentration measurement could identify gut inflammation in patients with symptomatic post-operative Crohn's disease. Patients had to have a histological diagnosis of Crohn's disease to be included in the study and had to have ileocaecal resection. The study period included patients who have had resection between January 1982 and August 2007. From the medical and surgical gastroenterology clinics at Newcastle upon Tyne Hospitals NHS Foundation Trust patients were identified for the study.

3.1.1 Aim:

This study involved investigation of two related arms: cross sectional and longitudinal studies. The Cross sectional study results address the following four aims of the study.

Quantitative measurement of faecal markers faecal calprotectin and faecal lactoferrin may offer a valuable method as non-invasive markers in assessing the symptomatic post-operative patient in Crohn's disease.

To investigate this hypothesis this study had the following aims:

1. To determine whether faecal calprotectin and faecal lactoferrin measurements in symptomatic post-operative Crohn's patient can differentiate those with aggressive disease from those with benign disease.
2. To compare faecal calprotectin and faecal lactoferrin with other measurements of inflammatory activity such as C-reactive protein,

white cell count, platelet count, clinical disease activity index (HBI) and endoscopic finding.

3. To determine how faecal calprotectin and faecal lactoferrin correlate with each other after resection.

3.2 Materials and Methods:

3.2.1 Clinical Data:

A total of 104 patients with a mean duration of 24 (range 2-300) months since surgery were recruited. All patients had undergone a previous ileocaecal resection for Crohn's disease. The following clinical characteristics were recorded from patient's notes and direct interview: age at disease or symptom onset, smoking history, sex, disease location, disease behaviour, pharmacology therapy and ileocolonoscopy findings (considered valid if performed within 4 weeks of patient interview). To estimate clinical disease activity Harvey Bradshaw Index Score was also calculated for each patient. A Score <3 indicated clinically inactive, 4- mildly active, 5-moderately active and >6 severely active disease. Venous blood was obtained for white cell count (normal range 4.3-10 $\times 10^9$/L), C-reactive protein (< 5miligram/L) and platelets (normal range 150-350 $\times 10^9$/L). On the same day a single stool sample for faecal calprotectin and lactoferrin was collected.

3.2.2 Stool sample collection:

This has been described in detail in material and methods chapter of this thesis.

3.3 Results:

3.3.1 Faecal calprotectin and faecal lactoferrin, blood parameters and Harvey Bradshaw Index clinical disease activity:

Clinical characteristics of the patients are shown in table-3.1. 140 patients were invited to participate in the cross sectional study but 23 were lost at the stage of stool collection. 104 patients participated in the cross sectional study with male to female ratio 43:61 and their median age at study was 45 (18-79) years and the remaining 13 patients are included in longitudinal study. The median follow up since ileocaecal resection is 12 (1-40) years. All patients had open ileo-colic resection since January 1999 and August 2007.

Table 3.1 - Demographic and clinical profile of cross sectional study group.

		Cross sectional
Total number of patients in study group		104
Sex (M:F)		43:61
Mean age in years (range)		45 (18-79)
Mean duration of disease in years (range)*		12 (1-40)
Time after ileocaecal resection in months (range)		24 (2-300)
Disease Location	Ileal	71
	Ileo-Colic	28
	Perianal disease†	5
Disease Behaviour	Inflammatory	17
	Stenosing	51
	Perforating	31
	Perianal Fistula†	5
Smoking Status	Never	31
	Ex-smokers	32
	Current	41
Endoscopy (< 1month at follow up)	Recurrence	25/43
	No recurrence	18/43
Maintenance Therapy	No medications	33
	5 – aminosalicylic	10
	Corticosteroids	23
	Azathioprine	39
	Methotrexate	11
	TNF- alpha antagonist	3

* Values are median (range). †Perianal disease present in combination with ileal or ileo-colic disease.

The mean (±SE) calprotectin levels in this cohort of patients with Crohn's disease was 300 µg/g (± 44.6), lactoferrin 12.02µg/g (± 12), CRP 19.15 mg/L (± 4.28), white cell count 8.51 mg/L (± 0.31) and platelet count 304 (± 8.23) as shown in table-3.2. Both faecal calprotectin and lactoferrin correlated significantly with the HBI definition of clinical disease activity ($r = 0.532$, $P< 0.001$, $r = 0.687$, $P< 0.001$ respectively). CRP level also showed a significant but weak correlation with current disease activity ($r = 0.329$; $P <0.001$). Based on Harvey Bradshaw index score, 28 patients had clinically active disease (HBI ≥6) and 43 had inactive (HBI≤3), 19 had mildly active (HBI = 4) and 14 patients had moderately active disease (HBI = 5). All patients with severely clinically active disease (28/104) had high faecal calprotectin level 661.1µg/g (±119.1), faecal lactoferrin level 116.6µg/g (±32.21), CRP 44.54mg/L (±14.34) compared with clinically inactive group {calprotectin 70.18 µg/g (±27.12), lactoferrin 5.92 µg/g (±2.39), CRP 7.72 mg/L (±1.67)} figure 3.1 and 3.2. The logarithmic distribution of faecal calprotectin and lactoferrin related to HBI is shown in Figure 3.3 and 3.4 respectively.

Table 3.2 - Correlation of faecal, serum parameters and Harvey Bradshaw Index clinical activity.

Clinical activity (HBI score) & Patients numbers (n=)	**Faecal Calprotectin levels (µg/g) Mean±SE**	**Faecal Lactoferrin levels (µg/g) Mean±SE**	**White cell count (×109/L) Mean±SE**	**CRP (mg/l) Mean±SE**	**Platelet count (×109/L) Mean±SE**
None (<3) n= 43	70.2(±27.1)	5.9(±2.4)	7.5(±0.3)	7.7(±1.7)	311.7(±13 .7)
Mild (4) n = 19	333.7(±78.9)	51.3(±14.8)	8.8(±0.8)	14.6(±5.2)	271.5 (±15.1)
Moderate (5) n = 14	242.4(±79.2)	77.8(±50.4)	8.4(±0.6)	9.7(±2.5)	310.3(±16.1)
Severe (>6) n = 28	661.1(±119.1)	116.6(±32.2)	9.9(±0.8)	44.5(±14.3)	31 1.0(±17.8)
P value Kruskal Wallis test	p=0.0001	p=0.0001	p=0.1085	p=0.0006	p=0.2769

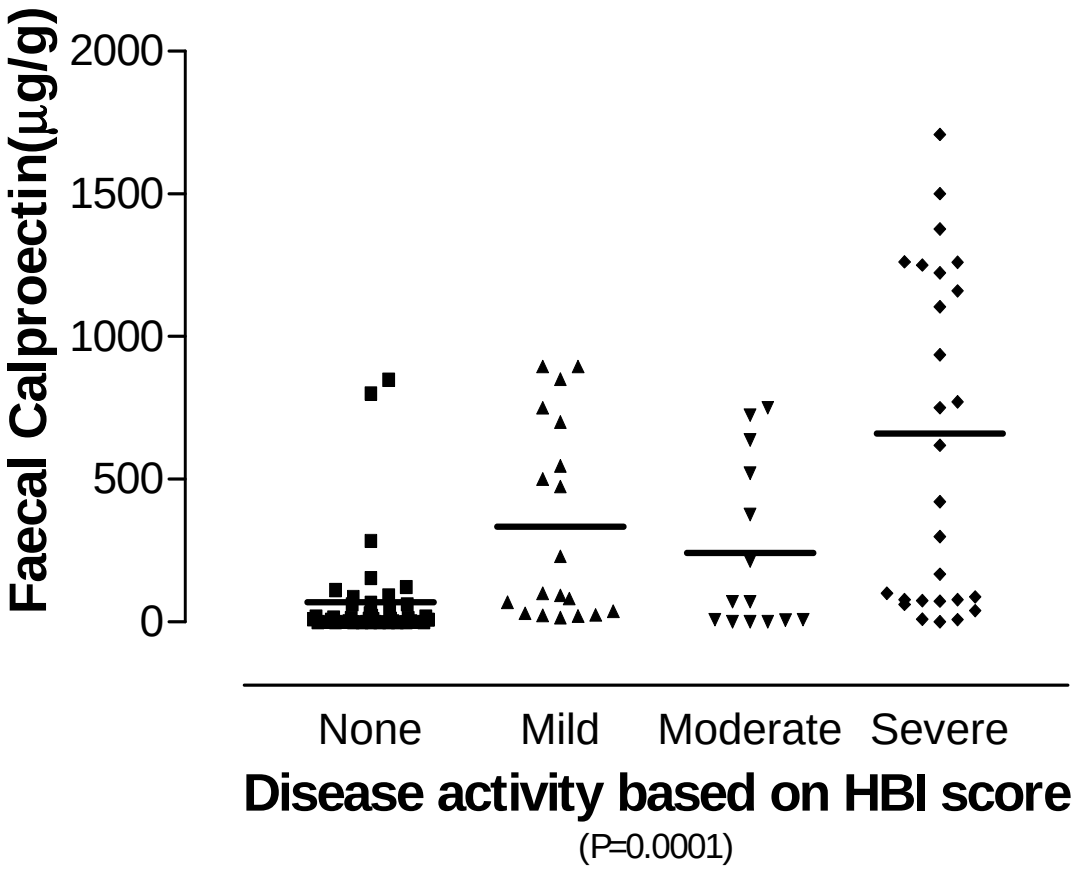

Figure 3.1 - Mean values of faecal calprotectin in correlation with HBI score. Horizontal bars represent mean values of faecal calprotectin.

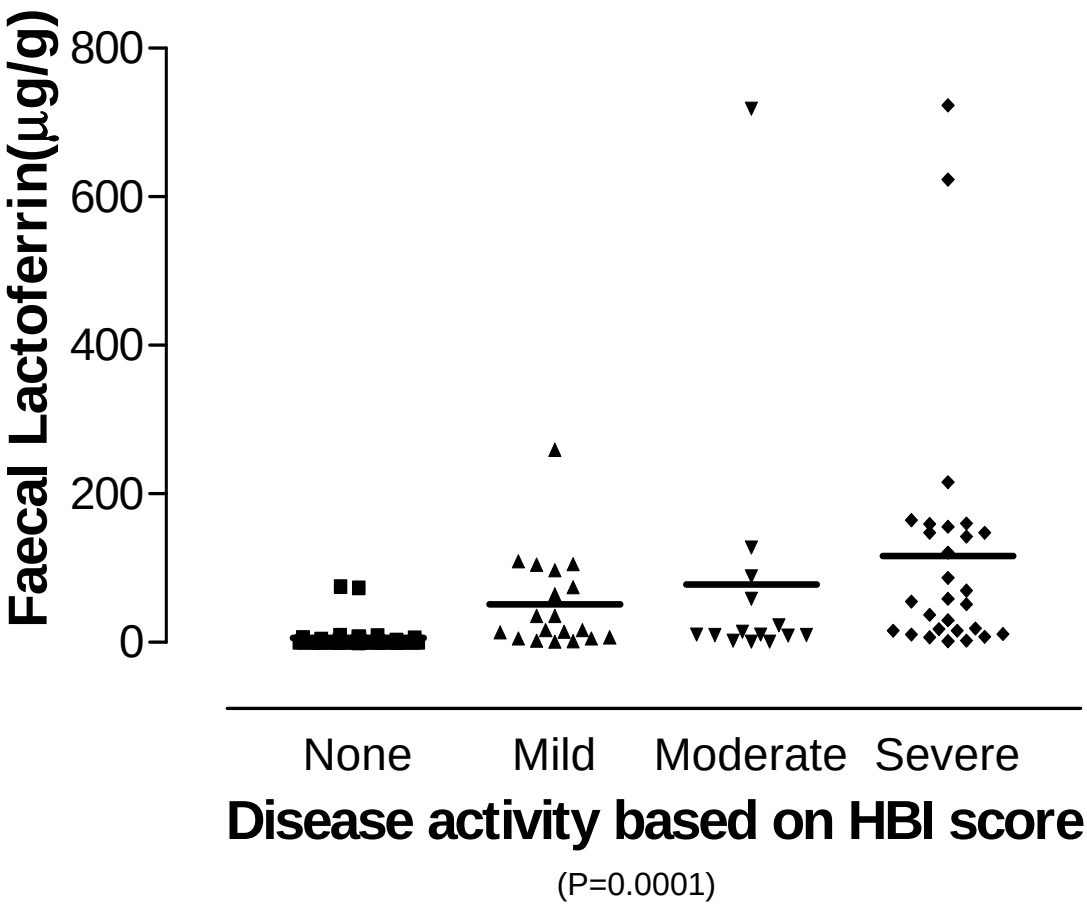

Figure 3.2 - Mean values of faecal lactoferrin in correlation with HBI score. Horizontal bars represent mean values of faecal lactoferrin.

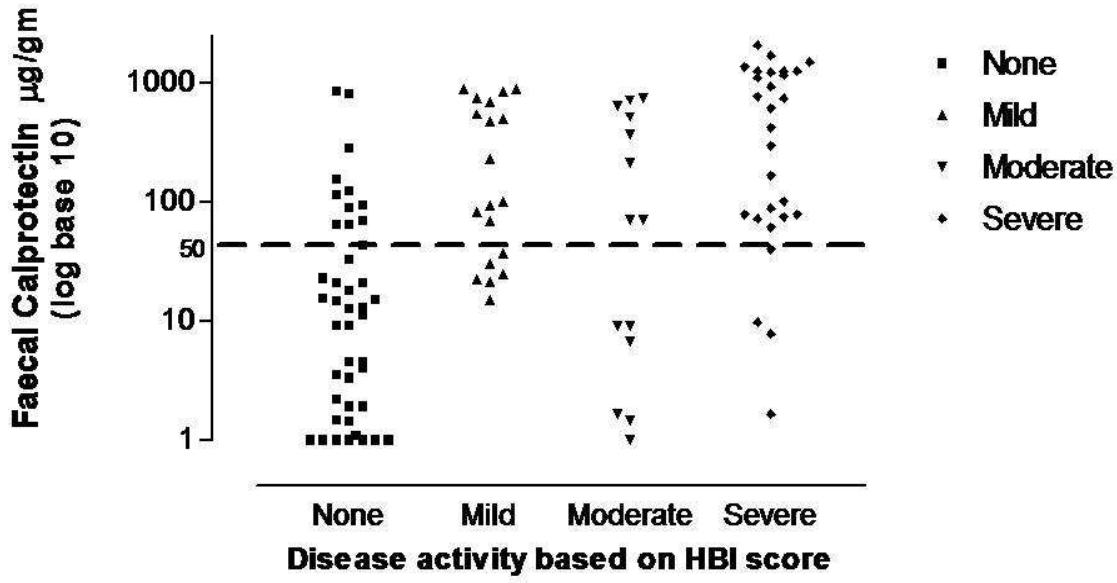

Figure 3.3 - Faecal calprotectin-logarithmic distribution related to HBI score. Horizontal line represent normal value of faecal calprotectin = 50 µg/g.

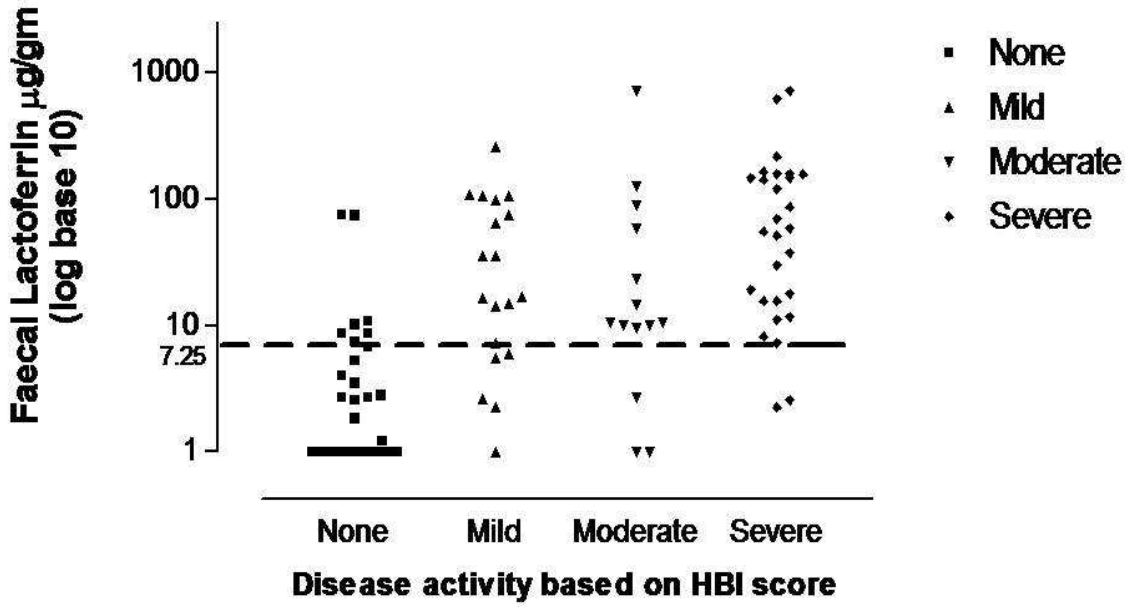

Figure 3.4 - Faecal lactoferrin-logarithmic distribution related to HBI score ($p<0.001$). Horizontal line represent normal value for faecal lactoferrin= 7.25 µg/g.

3.3.2 Heterogeneous Group:

The mean faecal calprotectin, lactoferrin levels and HBI were compared in high versus low levels of gut inflammation, which was arbitrarily defined by faecal calprotectin (100 µg/g) or lactoferrin (14.5 µg/g) values of more or less than twice (table-3.3) the upper limit of normal (ULN). Those who were clinically asymptomatic or had inactive disease (HBI≤3) had low mean faecal calprotectin and faecal lactoferrin values demonstrating inactive gut inflammation. Patients with high levels of clinically symptomatic or severely active disease (HBI≥6) tend to have higher levels of faecal calprotectin and faecal lactoferrin corresponding to highly active gut inflammation. Those with clinically mild to moderate disease activity (HBI 4-5) were far more heterogeneous. The patients having high or low levels of gut inflammation as defined above were almost equal in number. Thus faecal calprotectin and faecal lactoferrin allowed identifying two groups with very different biomarker levels of gut inflammation with similar clinical severity index.

In heterogeneous group (figure 3.5 and 3.6) of patients {Mild (19) Vs Moderate (14)} levels of faecal calprotectin, faecal lactoferrin, white cell count, platelet count and CRP were abnormal but did not correlate significantly with disease activity (table- 3.4).

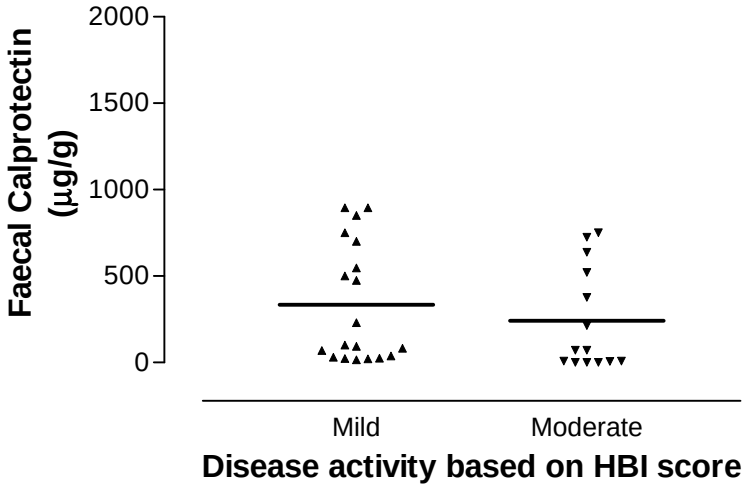

Figure 3.5 - Faecal calprotectin in heterogeneous group (mild & moderate).

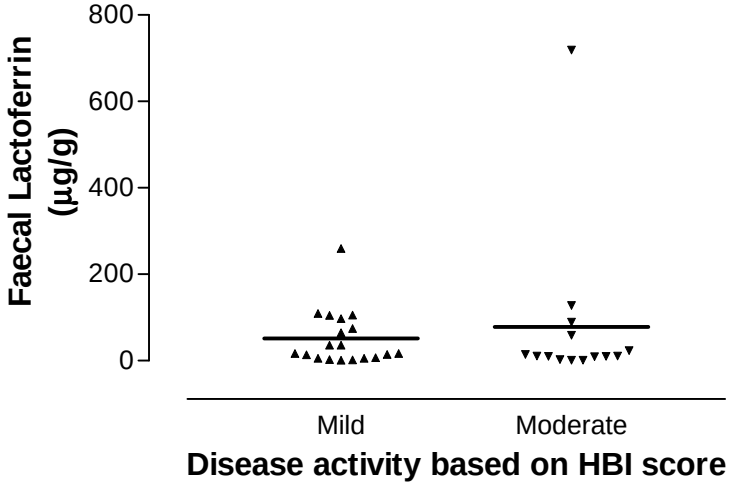

Figure 3.6 - Faecal lactoferrin level in heterogeneous group (mild & moderate).

Table 3.3 - Correlation between high and low levels of gut inflammation defined by faecal markers and Harvey Bradshaw clinically disease activity index.

	Pts n=104	**Faecal Calprotectin (FC µg/g)**	**Faecal Lactoferrin (FL µg/g)**	**HBI(≤3) n=43**	**HBI(4-5) n=33**	**HBI(≥6) n=28**
FC(<100 µg/g) 2 xULN	64	28	-	37	17	10
FC(>100 µg/g) 2 xULN	40	737	-	6	16	18
FL(<14.5 µg/g) 2 xULN	62	-	4	41	15	6
FL(>14.5 µg/g) 2 xULN	42	-	127	2	18	22

Table 3.4 - Heterogeneous group.

Disease Activity	**Mild (HBI=4) N=19**	**Moderate(HBI=5) N=14**	**p-value (Spearman's correlation coefficient)**
Faecal Calprotectin	333.7(±78.87)	242.4(±79.15)	p=0.138 (r = -.264)
Faecal Lactoferrin	51.28(±14.75)	77.8(±50.37)	p=0.497 (r = -.122)
White cell count($\times 10^9$/L)	8.81(±0.85)	8.43(±0.60)	p=0.901 (r = -.023)
CRP(mg/l)	14.58(±5.19)	9.71(±2.46)	p=0.557 (r = -.106)
Platelet count($\times 10^9$/L)	271.5(±15.08)	310.3(±16.08)	p=0.128 (r = -.271)

3.3.3 Faecal markers and endoscopic activity:

43 out of 104 patients had endoscopic assessment up to 4 weeks before the direct patient interview. Out of these, 25 had endoscopic proven disease recurrence. Mean (±SE) values (table-3.5) of white cell count, platelet count and CRP were not significant with absence of disease (P = 0.0815). There was no significant difference between mean (±SE) values of faecal calprotectin and lactoferrin with endoscopic disease recurrence and those without endoscopic disease recurrence (P=0.676 and P=0.73 respectively).

There was no significant Spearman's correlation between faecal calprotectin and faecal lactoferrin with no endoscopic recurrence (18/43) and with endoscopic recurrence (25/43) (figure 3.7 and 3.8).

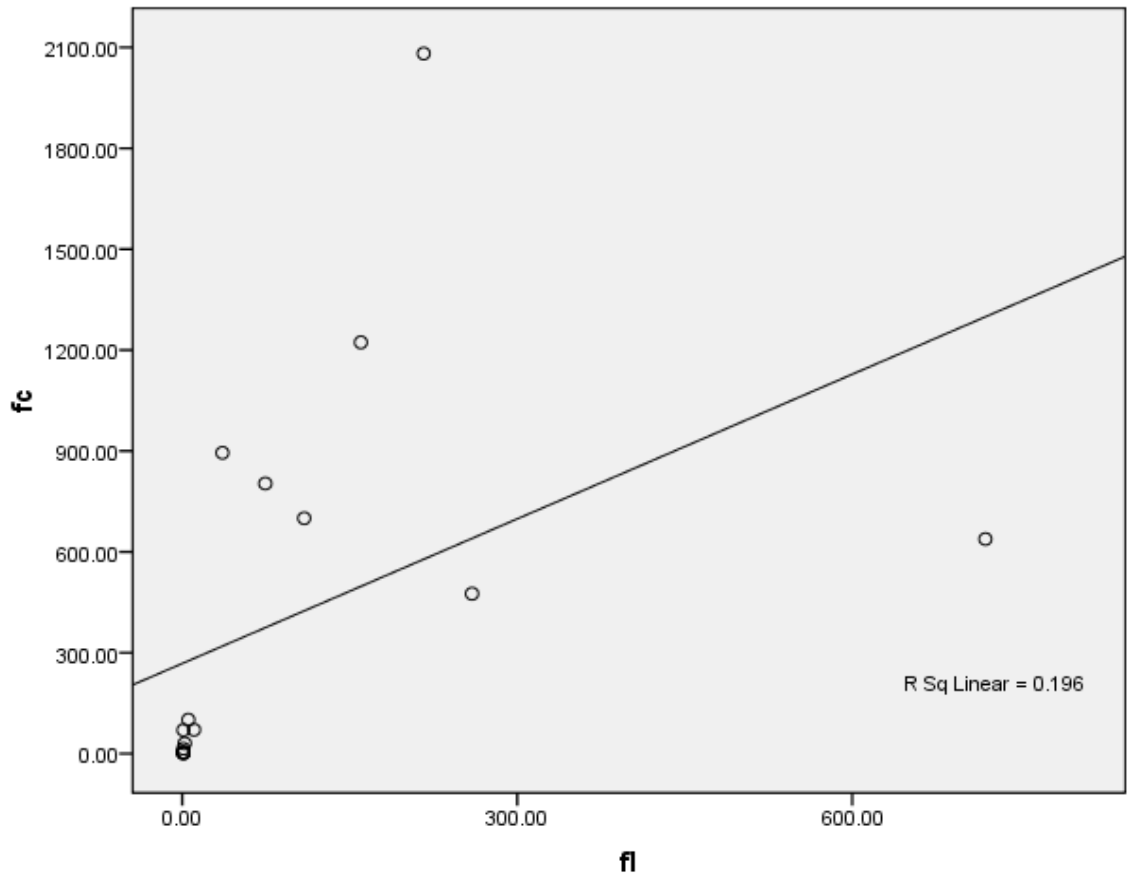

Figure 3.7 - Correlation of faecal calprotectin and lactoferrin with no endoscopic recurrence.

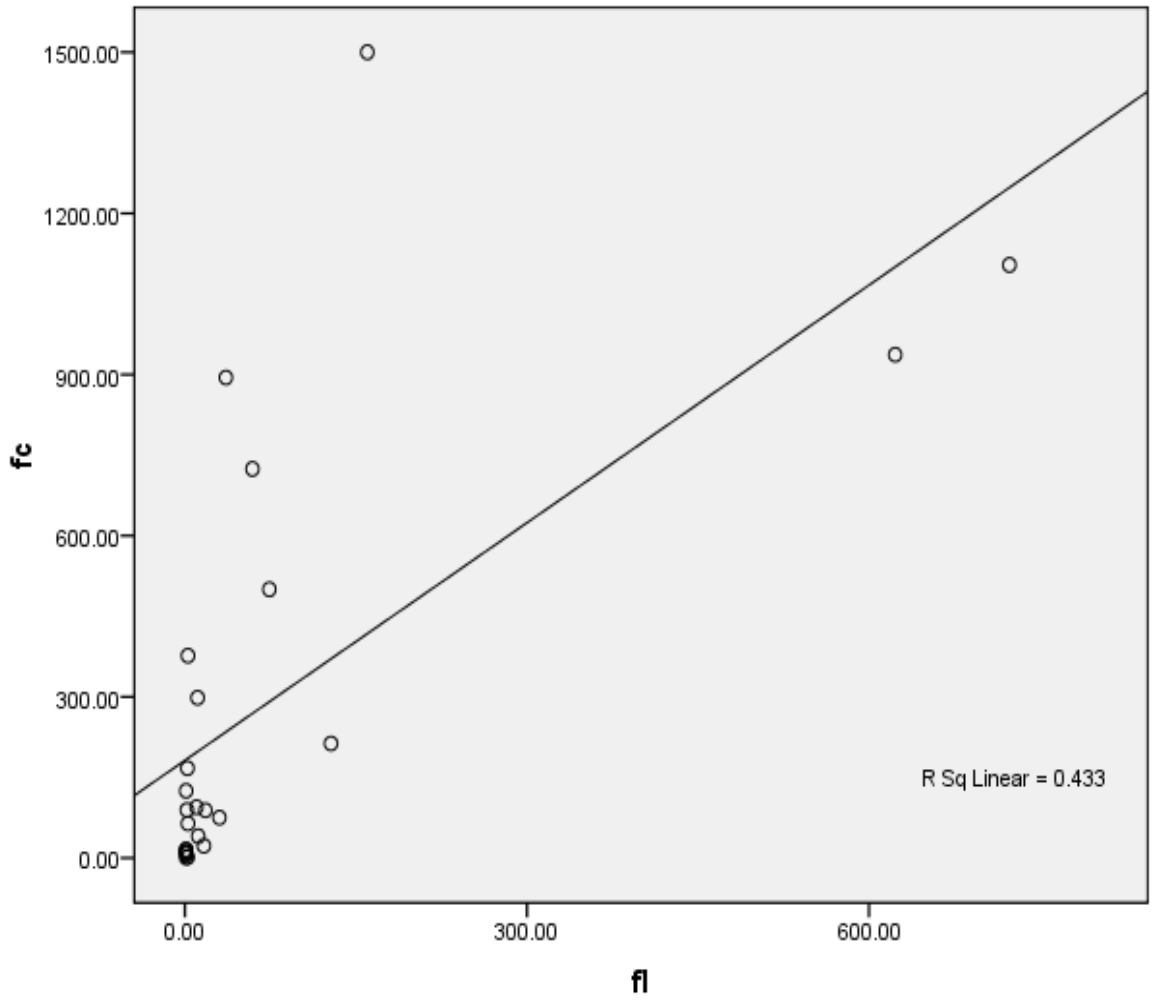

Figure 3.8 - Correlation of faecal calprotectin and lactoferrin with endoscopic recurrence.

Table 3.5 - HBI score, serum parameter and faecal marker levels in patients with and without endoscopic disease recurrence.

N=43/104	Endoscopic- no disease recurrence (18/43)	Endoscopic- disease recurrence (25/43)
Faecal calprotectin (µg/g) Mean±SE	295(±82.69)	395(±135.3)
Faecal Lactoferrin (µg/g) Mean±SE	76.81(±36.81)	88.91(±41.76)
Platelet count (×109/L) Mean±SE	325.9(±17.19)	331.8(±14.35)
White cell count (×109/L) Mean±SE	8.72(±.64)	8.61(±.59)
CRP (×109/L) Mean±SE	16(±5.86)	22.83(±11.93)
HBI score ≤ 3	9	8
HBI score 4-5	7	8
HBI score ≥ 6	2	9

Kruskal-Wallis test - p value = 0.0815

3.3.4 Faecal markers and smoking status:

Out of total 104 patients, 41 were current smokers, 32 former smokers and 31 who had never smoked. No significant difference was found between smokers and non smokers in the faecal calprotectin (P= 0.284) and faecal lactoferrin (P= 0.612) using Mann-Whitney U test.

3.3.5 Faecal markers and maintenance therapy:

The 104 patients included 23 patients who received corticosteroid therapy and 39 of which received azathioprine. There was no significant difference in the levels of faecal calprotectin, lactoferrin, white cell count, platelets and CRP between patients, who are on azathioprine (P=0.082), corticosteroids (P=0.159) and methotrexate (P=0.082).

3.3.6 Correlation between faecal calprotectin and faecal lactoferrin:

A total of 259 stool samples were used in both cross sectional (104) and longitudinal study (155). Using Spearman's correlation both faecal calprotectin and lactoferrin values showed significant correlation with each other ($r = 0.71$, $P < 0.001$) (figure 3.9).

Sub-group analysis in an individual group of clinical disease activity (none, mild, moderate and severely active, based on HBI) was performed in cross-sectional study. This was done to identify if any difference exists between faecal calprotectin and faecal lactoferrin in this individual group. Both faecal calprotectin and lactoferrin showed significant correlation with each other (figure 3.10, 3.11, 3.12 and 3.13). This show both faecal calprotectin and faecal lactoferrin were comparable with disease activity.

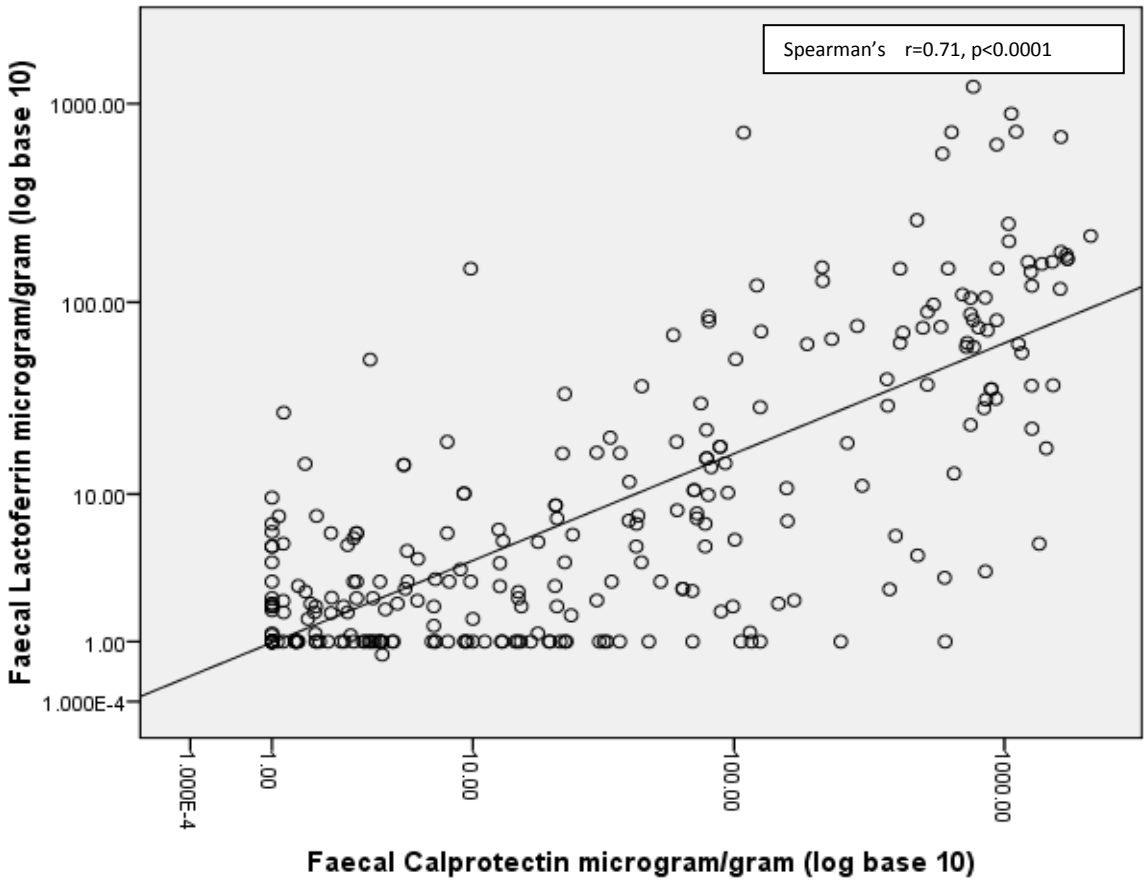

Figure 3.9 - Correlation of faecal calprotectin and lactoferrin in all stool samples.

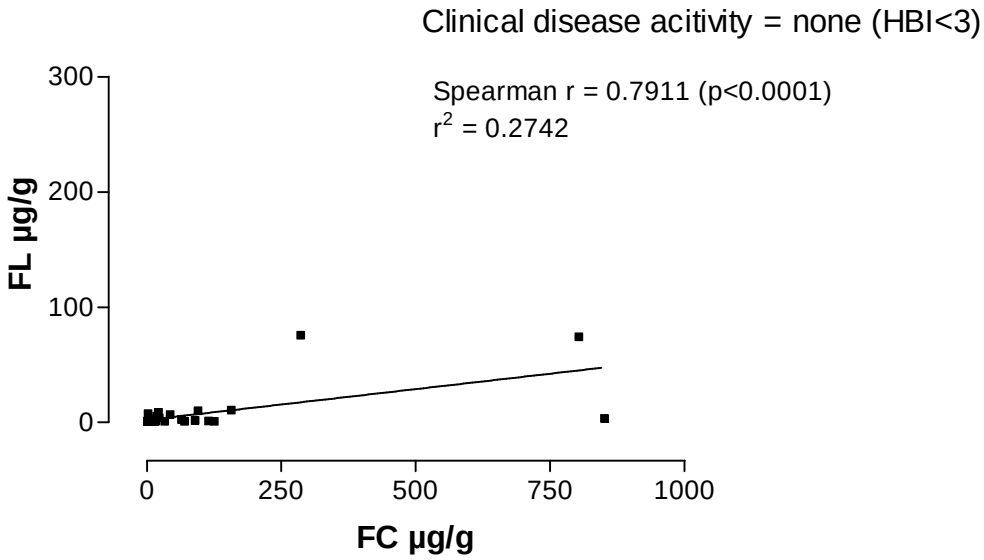

Figure 3.10 - Correlation of faecal calprotectin and lactoferrin in patients with HBI<3 (n=43).

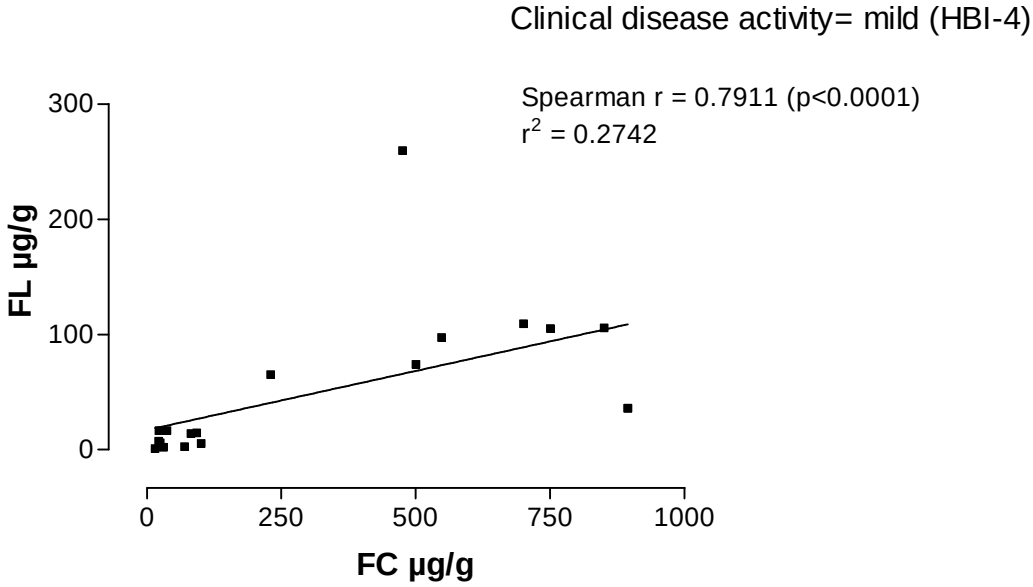

Figure 3.11 - Correlation of faecal calprotectin and lactoferrin in patients with HBI=4 (n=19).

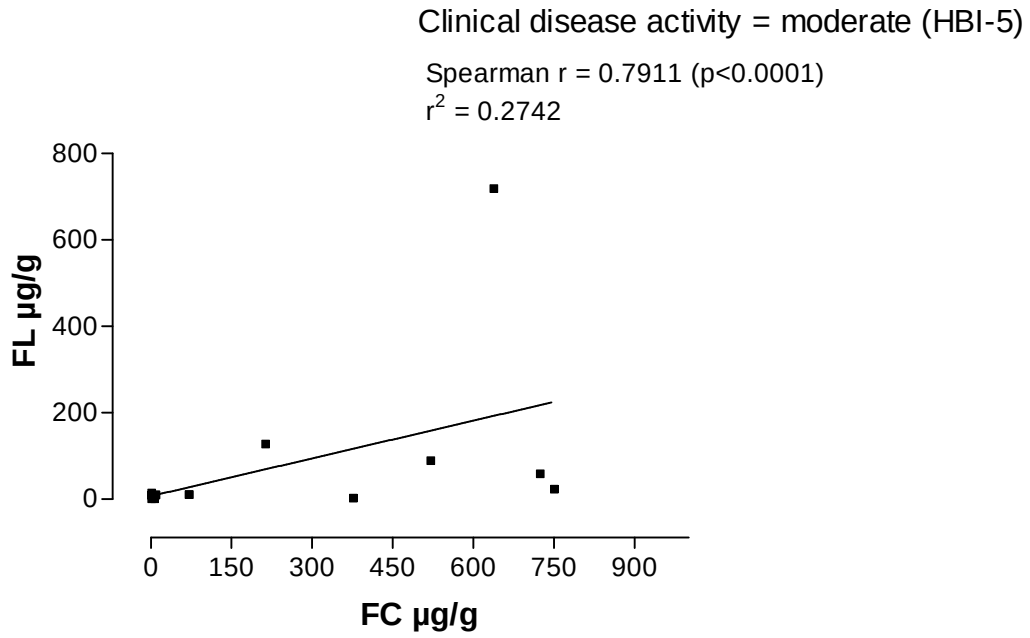

Figure 3.12 - Correlation of faecal calprotectin and lactoferrin in patients with HBI-5 (n=14).

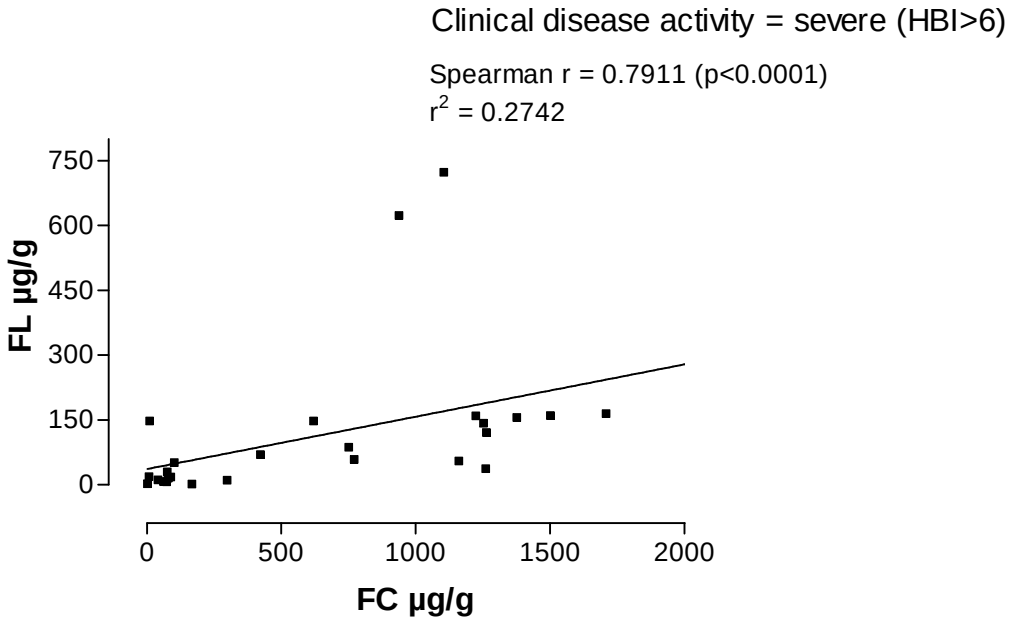

Figure 3.13 - Correlation of faecal calprotectin and lactoferrin in patients with HBI>6 (n=28).

Chapter 4

Longitudinal Study

Results

4.1 Introduction:

To determine whether faecal calprotectin and faecal lactoferrin concentration measurement could identify gut inflammation in patients with symptomatic post-operative Crohn's disease and to monitor the trend of postoperative faecal calprotectin and lactoferrin levels. Patients had to have a histological diagnosis of Crohn's disease to be included in the study and were going to have ileocaecal resection for symptomatic Crohn's disease. The study period included patients who went for ileocaecal resection between August 2006 and August 2007 and followed for 12 months post resection. From the medical and surgical gastroenterology clinics at Newcastle upon Tyne Hospitals NHS Foundation Trust patients were identified for the study.

4.1.1 Aim:

The longitudinal study result addresses the following aims of the study.

Quantitative measurement of faecal markers, faecal calprotectin and faecal lactoferrin may offer a valuable method as non-invasive markers in assessing the individual post-operative patient in Crohn's disease.

To investigate this hypothesis this study had the following aims:

1) To determine the immediate post-operative course of faecal calprotectin and faecal lactoferrin after ileocaecal resection.
2) To determine whether these faecal markers could demonstrate inflammatory activity and identify post-operative disease recurrence.

3) To identify if there is a difference in this two markers among uncomplicated and complicated group (with post-operative complications like anastomotic leak, intra-abdominal collection, wound infection and disease recurrence) of patients in immediate post-operative setting.

4.2 Materials and Method:

4.2.1 Clinical data:

A total of 13 patients who had undergone ileocaecal resection were followed for 12 months for symptomatic Crohn's disease. The following clinical characteristics were recorded from patient's notes and direct interview: age at disease or symptom onset, smoking history, sex, disease location, disease behaviour, current pharmacology therapy and ileocolonoscopy findings (considered valid if performed within 4 weeks of patient interview). Data on the complications, need for further interventions, postoperative course and outcome were collected. Serial stool samples including before surgery, at weekly intervals for 4 weeks and monthly intervals for 12 months were collected to determine the trends in faecal calprotectin and lactoferrin levels after surgery.

4.2.2 Stool sample collection:

This has been described in detail in material and methods chapter of this thesis.

4.3 Results:

4.3.1 Longitudinal study:

A group of 13 patients were included in this study. This group set up was to determine the course of faecal calprotectin and lactoferrin immediately after surgery. 155 stool samples were collected in this group. The demographic and clinical profile of longitudinal study group is shown in table – 4.1. Mean (± SE) pre-operative levels of both faecal calprotectin (879 µg/g ± 174.5) and lactoferrin (398.9 µg/g ± 164.5) were significantly high in all patients in this group.

Table 4.1 - Demographic and clinical profile of longitudinal study group.

		Longitudinal
Total number of patients in study group		13
Sex (M:F)		4:9
Mean age in years (range)		34 (18-64)
Mean duration of disease in years (range)		8 (1-30)
Disease Location	Ileal	10
	Ileo-Colic	3
	Perianal disease	1
Disease Behaviour	Inflammatory	3
	Stenosing	7
	Perforating	3
	Perianal Fistula	1
Smoking Status	Never	5
	Ex-smokers	3
	Current	5
Endoscopy (< 1month at follow up)	Recurrence	3/4
	No recurrence	¼
Maintenance Therapy	No medications	5
	5 – aminosalicylic	3
	Corticosteroids	4
	Azathioprine	8
	Methotrexate	2
	TNF- alpha antagonist	2

4.3.2 Uncomplicated post-operative recovery patients:

Uncomplicated group of patients are those patients without any post-operative complications like anastomotic leak or intra-abdominal collection or wound infection or disease recurrence. 8 of 13 patients had an uncomplicated postoperative recovery. Mean (± SE) of faecal calprotectin and lactoferrin were highest before surgery, at 562(155.7) µg/g and 347.7(159.0) µg/g respectively, but fell quickly after resection (table-4.2). Both markers normalised by 2 months and remained within normal limits during the entire study period. Both faecal calprotectin and lactoferrin levels variation with time was similar in each patient. The individual trends and median values for each patient are shown in figure 4.1, 4.1a, 4.2 and 4.2a. Out of these 8 uncomplicated patients only one patient had an unexplained increase in faecal calprotectin and lactoferrin level. This patient had a high pre-operative level of 1035.8µg/g and 248.7µg/g respectively, which normalised by two weeks and increased to 61µg/g and 19.1µg/g before spontaneously normalising at 12 months.

Table 4.2 - Faecal calprotectin and lactoferrin levels before and after resection in 8 patients with an uncomplicated recovery.

	Faecal Calprotectin **Mean(± SE) (µg/g)**	**Faecal lactoferrin** **Mean(± SE) (µg/g)**
Before Surgery	562.0 (155.7)	347.7 (159.0)
1 week	334.3 (114.2)	91.1 (68.0)
4 weeks	20.8 (12.4)	2.7 (0.7)
2 months	8.7 (2.8)	3.4 (0.7)
3 months	4.7 (2.7)	2.1 (0.7)
6 months	3.0 (0.9)	3.0 (0.8)

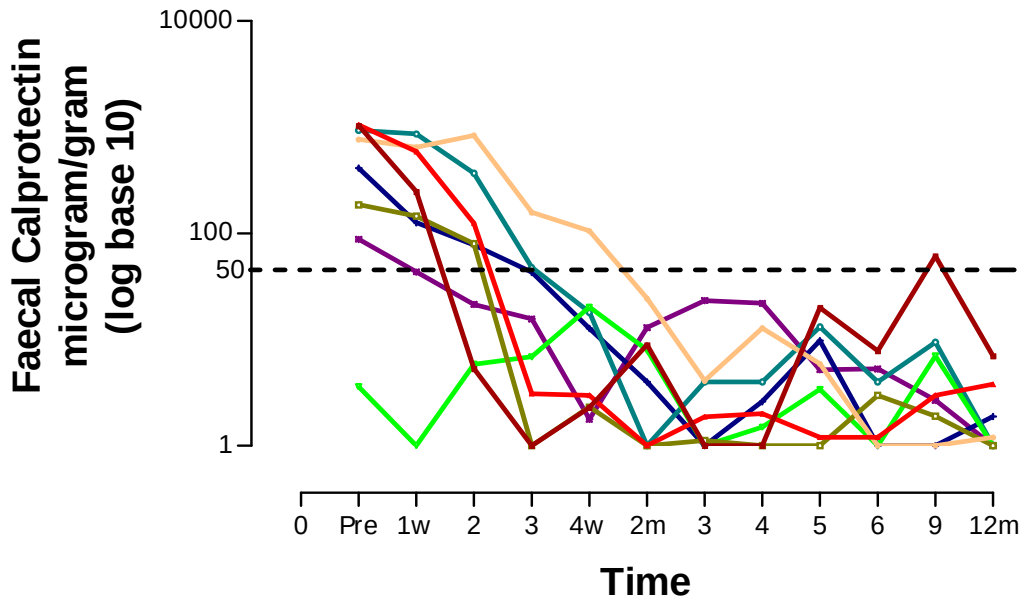

Figure 4.1 - Faecal calprotectin levels (individual values) pre-operative and at post-operative follow up of 8 patients with an uncomplicated post-operative recovery.

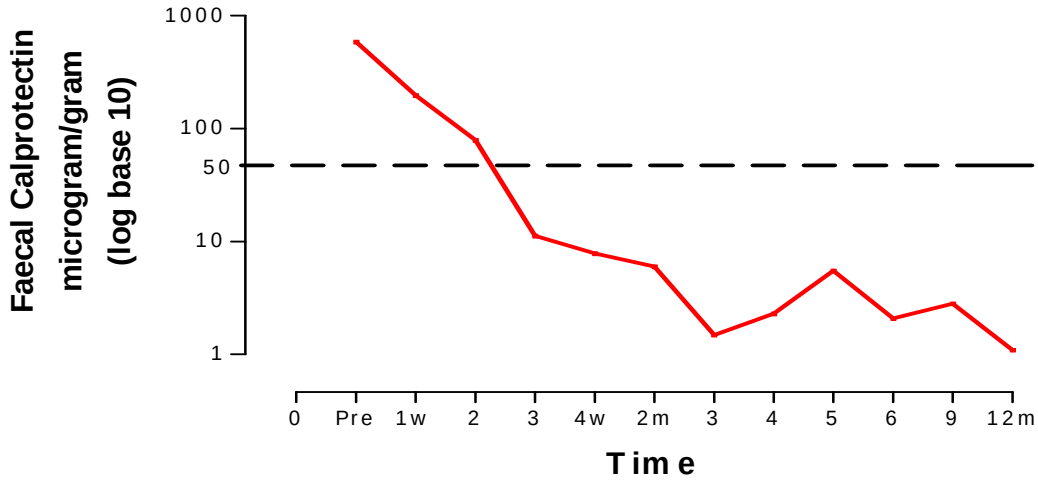

Figure 4.1a - Faecal calprotectin levels (median values) pre-operative and at post-operative follow up of 8 patients with an uncomplicated post-operative recovery.

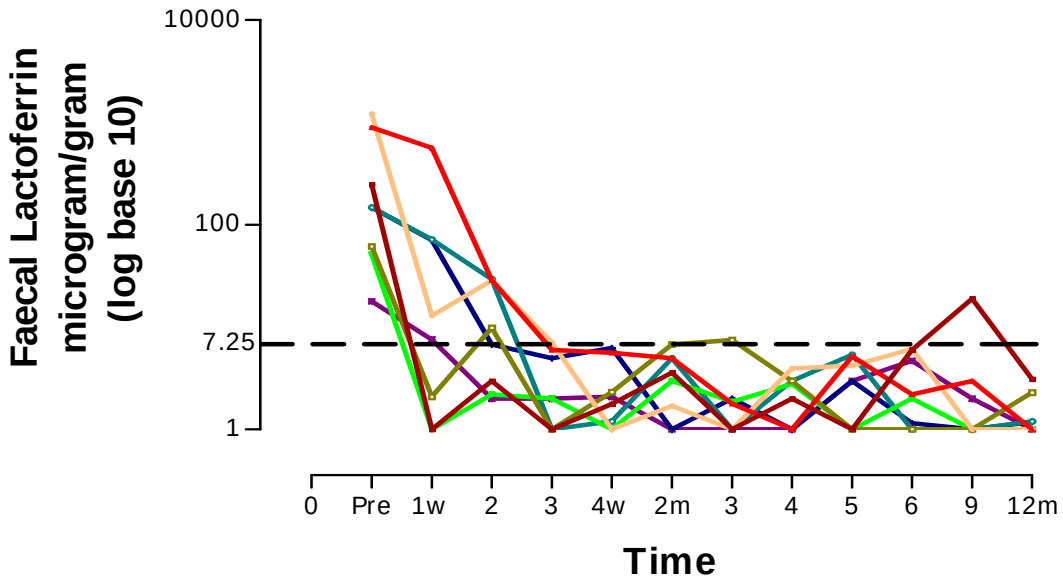

Figure 4.2 - Faecal lactoferrin levels (individual values) pre-operative and at post-operative follow up of 8 patients with an uncomplicated post-operative recovery.

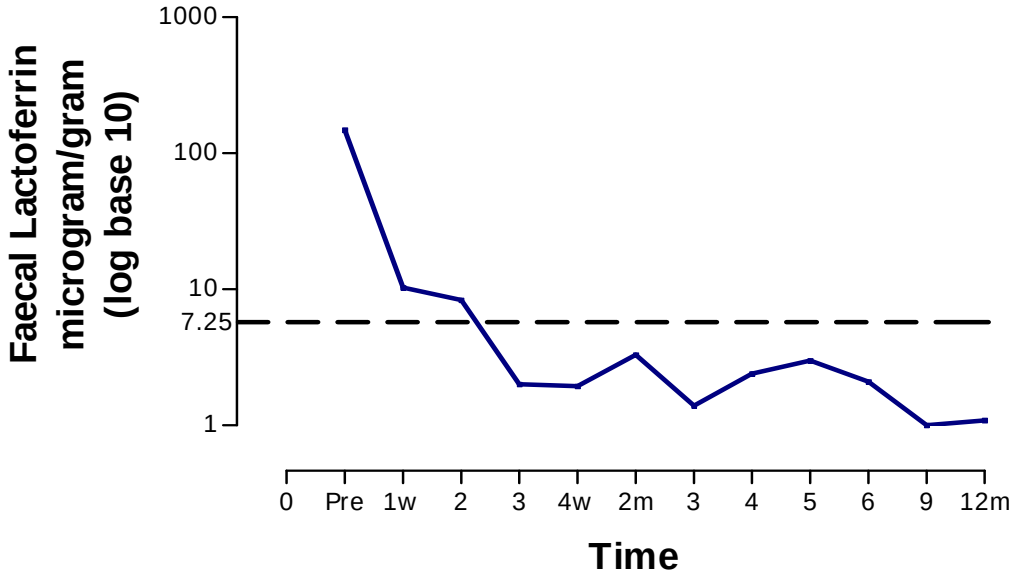

Figure 4.2a - Faecal lactoferrin levels (median values) pre-operative and at post-operative follow up of 8 patients with an uncomplicated post-operative recovery.

4.3.3 Complicated post-operative recovery patients:

Complicated patients are those patients with post-operative complications like anastomotic leak or intra-abdominal collection or wound infection and disease recurrence. 5 out of 13 patients had complications following ileocaecal resections (figure 4.3, 4.3a, 4.4 and 4.4a). Individual patient profile for the 5 complicated post-operative recovery patients is shown in table 4.3. A 58 year old male Patient A developed an intra-abdominal abscess one month after ileocaecal surgery. This patient had a pre-operative faecal calprotectin level of 519.8 µg/g. This quickly normalised but increased to 263.1µg/g at one month coinciding with leak after surgery. A pre-operative faecal lactoferrin level of 37.9µg/g normalised but then increased by the time of complication to 121.5µg/g. After surgical repair both these markers normalised within one month.

Second Patient B was a 69 year old female who developed a leak and intra-abdominal collection in the 2nd week after ileocaecal resection, underwent a laparotomy for drainage and made a good recovery. At the time of her complication her faecal calprotectin level climbed from 30.5µg/g to 1616µg/g and her faecal lactoferrin level increased from 5.8µg/g to 680.8µg/g. Both markers normalised one month after drainage of abscess.

Third Patient C was a 25 year old female who developed clinical and endoscopic recurrence as well as wound infection within one month of ileocaecal resection. Her preoperative faecal calprotectin level was

1616.8µg/g which fell slightly to 1344.5µg/g and then increased to 1607.0µg/g when she relapsed. Her preoperative faecal lactoferrin level of 179.5µg/g normalised after surgery to 5.2µg/g and then increased to 116.8µg/g at the time of relapse. After she was started on azathioprine and treatment for a wound infection, faecal markers normalised over three months time.

Fourth Patient D was a 21 year old male patient who had relapsed at 9 month, his pre-operative faecal calprotectin (1127.4µg/g) and lactoferrin (61.2µg/g) levels normalised by one month and at the time of his symptoms flare up went up to 78.9µg/g and 22.1µg/g needing azathioprine therapy. His levels had been consecutively high from month five onwards.

Fifth Patient E was a 34 year old male patient who had ileocolonic and perianal disease. His pre-operative faecal calprotectin and lactoferrin levels were 1707.6µg/g and 165.8µg/g, these normalised at first month post-operatively. Both his faecal calprotectin and lactoferrin climbed to 78.9µg/g and 22.1µg/g at 9th month as he relapsed and was started on infliximab. And at 12 months his markers normalised.

Table 4.3 – Individual patient profile for the 5 complicated post-operative recovery patients.

Patient	1	2	3	4	5
Age / Gender	58 M	69 F	25 F	21 M	34 M
Nature of complication	Collection	Collection	Disease relapse	Disease relapse	Disease relapse
Time of complication post op	1 month	2 weeks	1 month	9 months	9 months
Treatment	Surgery	Surgery	Azathioprine	Azathioprine	Infliximab
Pre op FL (μg/g)	37.9	1960	179.5	61.2	165.8
Time to FL normalization post op	2 weeks	2 weeks	1 week	3 weeks	2 weeks
Pre complication FL (μg/g)	3.2	5.8	5.2	4	6
FL at time of complication (μg/g)	121.5	680.8	116.8	22.1	150
FL following treatment of complication(μg/g)	1	1	-	1	6
Time to normalization following complication	2 months	5 weeks	-	3 months	3 months

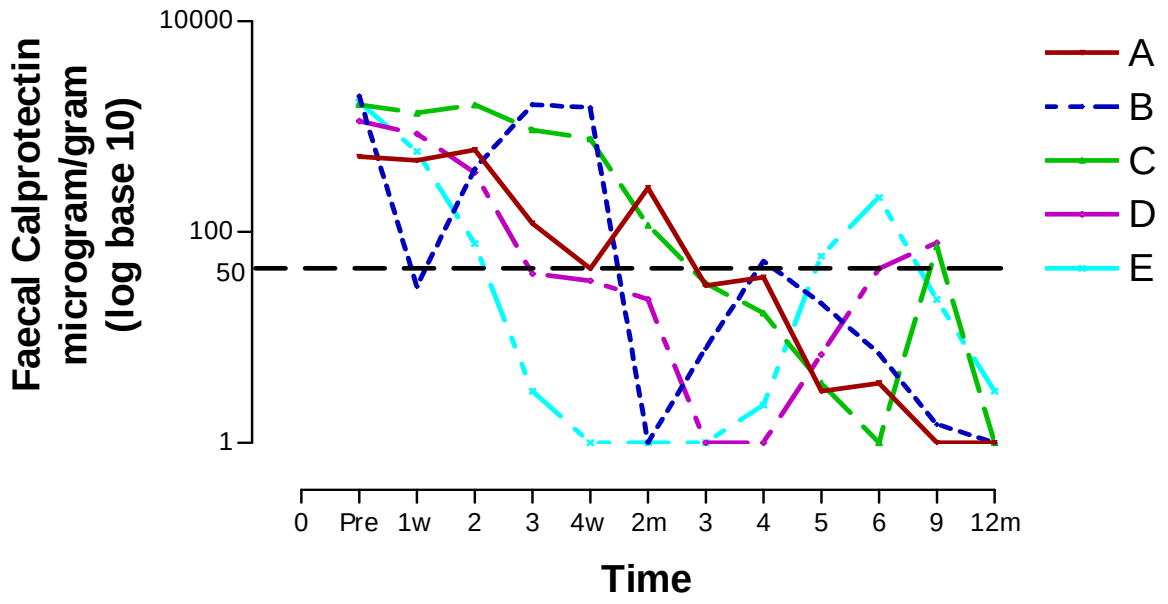

Figure 4.3 - Faecal calprotectin concentration (individual values) preoperatively and at post operative follow up for the 5 patients with complicated post-operative recovery.

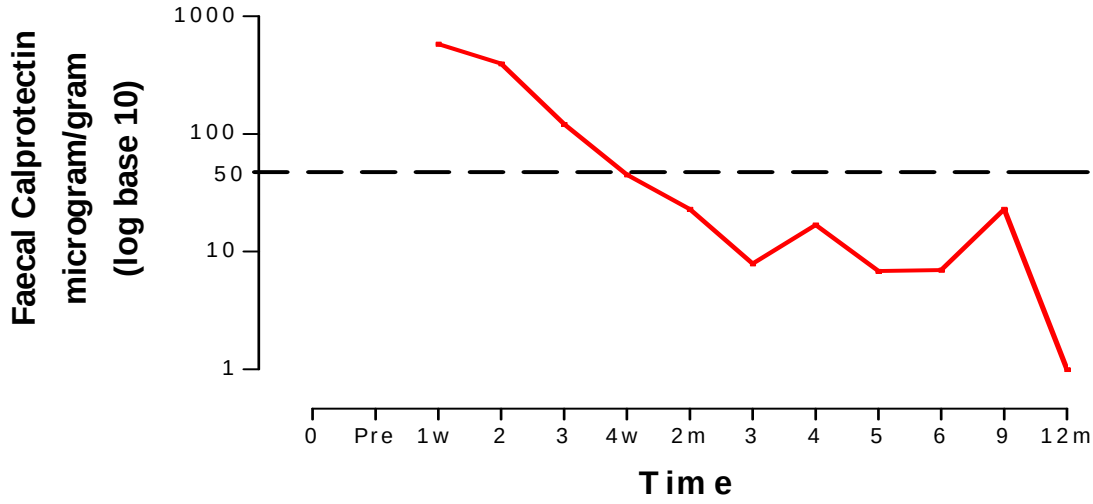

Figure 4.3a - Faecal calprotectin concentration (median values) preoperatively and at post operative follow up for the 5 patients with complicated post-operative recovery.

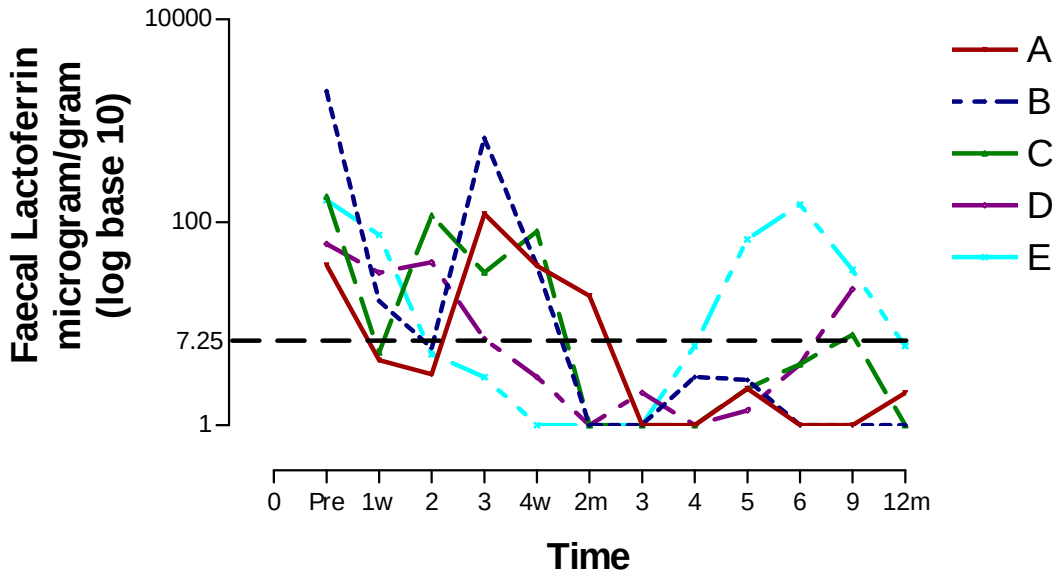

Figure 4.4 - Faecal lactoferrin concentration (individual values) preoperatively and at post-operative follow up for the 5 patients with complicated post operative recovery.

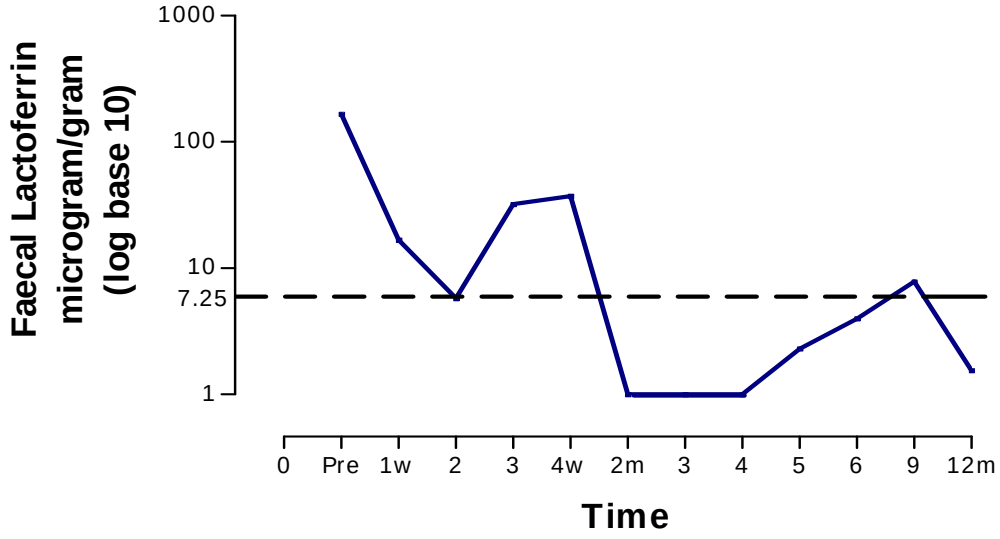

Figure 4.4a - Faecal lactoferrin concentration (median value) preoperatively and at post-operative follow up for the 5 patients with complicated post operative recovery.

Chapter 5

Discussion

5.1 Discussion on faecal markers:

This study (Cross-sectional and Longitudinal) has shown that the quantitative measurement of faecal calprotectin and faecal lactoferrin offer a valuable non-invasive method of assessing symptomatic post-operative patients with Crohn's disease. Both faecal calprotectin and faecal lactoferrin tests have been demonstrated as reproducible and correlated well with one another. After uncomplicated ileocaecal resection for Crohn's disease, both faecal calprotectin and faecal lactoferrin normalised in 2 months. Both remained within normal range (faecal calprotectin < 50µg/g and faecal lactoferrin < 7.25µg/g) unless there was a recurrence of gastrointestinal inflammation.

A previous report by Roseth et al [150] demonstrated that normalization of faecal calprotectin was associated not just with clinical remission but mucosal healing. Findings from this study (figure 4.1a and 4.2a), could suggest that in symptomatic post-operative patients, a single faecal calprotectin or faecal lactoferrin measurement at 2 months or more after ileocaecal resection could identify genuine disease recurrence and help to target immunosuppressant therapy. In patients who have had ileocaecal resection where the levels of faecal calprotectin or faecal lactoferrin remained at low levels, but have symptoms, are unlikely to have mucosal inflammation. The symptoms in this patient group may be due to altered anatomy, bile salt malabsorption or functional bowel disease. In this group of patients conservative management using anti-motility drugs such as

loperamide and cholestryamine should be considered. In contrast symptomatic patients, who had ileocaecal resection with high levels of faecal calprotectin or faecal lactoferrin, could have higher degree of mucosal inflammation. This group of symptomatic patients may benefit from agents such as thiopurines or anti-TNF- alpha antibodies. From this study, it has not been demonstrated that faecal calprotectin test is superior to faecal lactoferrin and neither can be used reliably as a single measurement to quantify gastrointestinal inflammation in post-operative Crohn's disease and vice-versa.

Among the various serological markers such as C-reactive protein, platelet count stands out for assessment of intestinal inflammation and correlation with symptoms and disease activity index [166]. These markers have low sensitivity and specificity for gastrointestinal inflammation and poorly correlate with symptoms and disease activity indexes. In this study both faecal calprotectin and faecal lactoferrin correlated more strongly with disease symptoms scoring according to HBI than CRP and platelet count (table 3.2). These faecal markers may therefore give more accurate reflection of gastrointestinal inflammation than the more commonly used serological markers. Similar findings to our study were seen in a study from Langhost et al [144]; they showed faecal calprotectin and faecal lactoferrin to be superior to C-reactive protein in overall assessment of gastrointestinal inflammation and correlation with clinical disease score.

Sipponen et al [172] and colleagues concluded that for evaluation of Crohn's disease activity based on endoscopic findings, faecal calprotectin and faecal lactoferrin are more sensitive markers. Surprisingly in our study, no significant difference was noticeable in faecal calprotectin or faecal lactoferrin concentration between patients with endoscopic evidence of gastrointestinal inflammation and those with normal endoscopic findings.

A study by D'Inca et al [130] was conducted to evaluate the efficacy of faecal calprotectin and faecal lactoferrin in detecting organic disease as assessed by colonoscopy. This study involved 144 patients undergoing colonoscopy for lower gastrointestinal symptoms or inflammatory bowel disease activity or surveillance for dysplasia. They concluded a significant correlation between colonic inflammation at endoscopy and faecal calprotectin, and between histological inflammation and faecal lactoferrin but not vice versa. In our study using faecal calprotectin and faecal lactoferrin as markers in post-operative recurrence in Crohn's disease surprisingly, no significant difference was apparent in faecal calprotectin and faecal lactoferrin levels between patients with endoscopic evidence of inflammation and those with normal endoscopic finding [1]. D'Inca et al recommended adjusting the cut-off values of both faecal calprotectin and faecal lactoferrin in the IBD group. However it should be noted that D'Inca and colleagues used a quantitative assay for faecal calprotectin but only a qualitative assay for faecal lactoferrin in contrast to our study where both

quantitative assay were used for both faecal calprotectin and faecal lactoferrin.

Previous research in patients with Ulcerative Colitis has suggested that faecal calprotectin correlates with the degree of inflammation rather than disease extent and the levels of faecal calprotectin are significantly higher in active small bowel Crohn's disease [149]. Therefore it may be that patients with high faecal calprotectin or faecal lactoferrin levels but normal endoscopy have active small bowel disease beyond the reach of colonoscope. It is also worth noting that the time between colonoscopy and interview in the present study was up to 4 weeks. The longitudinal data result demonstrated that there can be significant variation in faecal calprotectin and faecal lactoferrin levels within this time; hence when comparing faecal calprotectin and faecal lactoferrin levels with endoscopic finding, the interval between the two is a limitation.

Among various serological markers available like ESR, CRP, ANCA, platelet count and ASCA only CRP stands out in literature for assessing gastrointestinal inflammation and correlates with symptom and disease activity index [166]. However, CRP has low sensitivity and specificity and correlates poorly with gastrointestinal inflammation and disease activity index in IBD compared to faecal calprotectin and lactoferrin [4]. The current study has also demonstrated that both faecal calprotectin and lactoferrin correlate more strongly than CRP, WCC or platelet count with disease

symptoms scoring according to Harvey Bradshaw Activity Index [1], and may therefore give a more accurate reflection of gastrointestinal inflammation than these more commonly used serological markers.

In patients who had a complicated (intra-abdominal collection, wound infection, recurrence of disease) post-operative recovery, their both faecal calprotectin and lactoferrin increased above normal and normalized again after they responded to therapy. Burdeus and co-workers have demonstrated that in a paediatric population with Crohn's disease faecal lactoferrin could be useful in monitoring response to infliximab therapy when compared with the paediatric CDAI [183]. Kolho and colleagues[151] have shown that, although faecal calprotectin decreases in line with clinical response in patients with active IBD treated with corticosteroids, it does not always normalize. A likely explanation is the fact that corticosteroids do not induce the full resolution of mucosal inflammation [52] which could be achieved by other therapeutic means such as azathioprine or infliximab therapy [106, 107, 190].

The most novel finding of this study was the association between faecal calprotectin and faecal lactoferrin concentrations and long-term active and inactive disease in post-operative patients. The cross-sectional study showed a high degree of correlation between clinical disease activity and high and low levels of intestinal inflammation defined by a faecal calprotectin or faecal lactoferrin more or less than twice the upper limit of normal. In patients with no clinical disease activity (HBI score of less than

3), most patients had low levels of both faecal calprotectin and lactoferrin (less than twice the upper limit of normal), and therefore low levels of intestinal inflammation. Those with very active disease (HBI score of 6 or more) generally had high levels of gut inflammation (faecal calprotectin and faecal lactoferrin both over twice the ULN- table 3.3). However, in the heterogeneous group of patients (those with mild or moderate symptoms of disease activity {HBI score 4-5} formed a far more diverse group. This is important to recognize, as often these are the most challenging patients in which to distinguish inactive from active disease. The finding of an almost equal split between patients with high and those with low levels of gut inflammation within a similar spectrum of clinical disease suggests that a single measurement of faecal calprotectin and faecal lactoferrin could allow treatment targeting between immunosuppression for those with high concentrations of faecal granulocyte degradation products and symptomatic treatment for those with low concentrations.

This evidence favouring the routine use of faecal granulocyte markers in post-operative Crohn's disease is supported by the work of Orlando and co-workers [158]. They investigated the role of faecal calprotectin measurement, ultrasound (both performed at third month after surgery) and colonoscopy (performed at one year after surgery) predicting disease recurrence in patients with asymptomatic Crohn's disease within a year of resection, finding that a cut-off value of faecal calprotectin at 200 µg/l gave a sensitivity of 63% and specificity of 75%. They therefore recommended that

this value could be used as a tool to decide which patients should have colonoscopy after surgery.

Another cross-sectional study was conducted in post-operative Crohn's disease setting by Scarpa and colleagues [155] in a small cohort to assess faecal calprotectin and faecal lactoferrin concentrations randomly at a median follow up of 40.5 months after ileocolonic resection surgery. Using the Crohn's disease activity index (CDAI), they too demonstrated that some patients have a high faecal calprotectin and lactoferrin concentrations after surgery even in clinical remission. However, because their cohort was smaller than the present study, they could not stratify clinical disease activity, nor compare faecal calprotectin and faecal lactoferrin measurements. Furthermore, they did not have a cohort of patients with serial measurements of faecal calprotectin or lactoferrin from the time of surgery.

5.2 Conclusions:

This study has shown that quantitative measurements of faecal calprotectin and faecal lactoferrin offers a valuable, non-invasive method of assessing symptomatic postoperative patients with Crohn's disease. Both this tests have been demonstrated as reproducible and correlated well with one another. Compared to other measurements of inflammatory activity both these markers are better in identifying clinical disease activity. After uncomplicated ileocaecal resection for Crohn's disease, both markers normalized by two months [1]. Faecal calprotectin and lactoferrin measurement can be recommended for routine use in post-operative patients to help target immunosuppressant therapy. With this study using these two non-invasive markers, a proposed algorithm for monitoring & treatment after ileocaecal resection for Crohn's disease is shown below (figure 4.5).

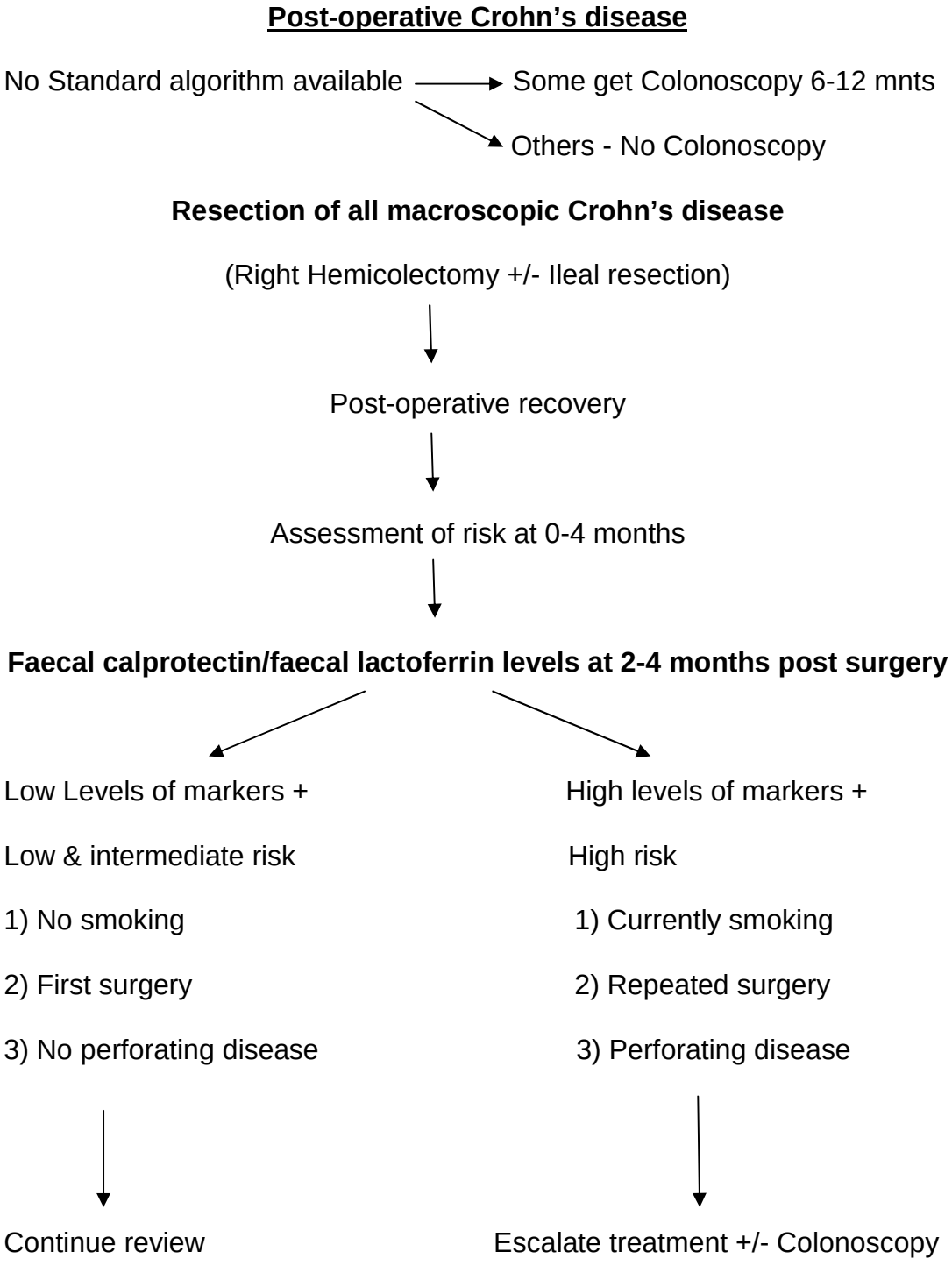

Figure 4.5 – Proposed algorithm for monitoring & treatment for Crohn's disease after Ileocaecal resection.

5.3 Limitation of the study:

The limitation of the study in this group of post-operative patients is the lack of a 'gold standard' test of disease recurrence. A colonoscopy for each patient would have been ideal, but still incomplete assessment of the Crohn's disease. The degree of testing would not be in the patient's interest as it is too invasive. It is therefore hard to validate the use of any test fully. There is also a degree of subjectivity and bias associated with the use of clinical indices such as the Crohn's Disease Activity Index and Harvey Bradshaw Activity Index, although clinical indices do reflect individual well-being, which in clinical practice is used to guide management. 13 patients in the longitudinal study are smaller than ideal and shorter follow up of 1 year. This study was designed to be achievable in the time frame and within budget available as well as acceptable to patients by virtue of not involving any invasive tests.

5.4 Future prospects:

In summary, the results from this study are encouraging for the use of faecal granulocyte degradation product markers in the post-operative surveillance of Crohn's disease patients and should stimulate larger randomized prospective trials evaluating if disease monitoring by faecal markers alters clinical outcomes in the long term follow up.

Future trials are desirable to investigate targeted azathioprine or anti-TNF therapy in those with high faecal calprotectin or faecal lactoferrin levels, and

withdrawing or reducing immunosuppressant therapy in those with low faecal calprotectin or faecal lactoferrin levels.

Already research and development and research ethics committee have approved a pre-pilot longitudinal study (to be conducted at Newcastle upon Tyne Hospitals NHS foundation trust and Newcastle University) to monitor fluctuations and trends in faecal calprotectin and lactoferrin, in a cohort of patients treated with anti-TNF antibody therapy over a 6 month time period. The hypothesis of future study is that prior to a symptomatic flare up of Crohn's disease, there will be a period of time where a rise in faecal biomarkers from baseline can be observed without patient's experiencing a clinical change. This period of subclinical disease activity may be an ideal target for therapeutic intervention. There is a large national study (TOPPIC) from Edinburgh in post-operative setting on Crohn's disease is underway using faecal calprotectin and faecal lactoferrin. This study involves collecting 290 patients prospectively for 2-3 years and help target immunosuppression therapy. This will add additional information about post-operative Crohn's disease apart from my thesis and expanded data will give further evidence for change of practice.

References

References:

1. Lamb, C.A., et al., *Faecal calprotectin or lactoferrin can identify postoperative recurrence in Crohn's disease.* Br J Surg, 2009. **96**(6): p. 663-74.
2. Angriman, I., et al., *Enzymes in feces: useful markers of chronic inflammatory bowel disease.* Clin Chim Acta, 2007. **381**(1): p. 63-8.
3. Tibble, J.A. and I. Bjarnason, *Fecal calprotectin as an index of intestinal inflammation.* Drugs Today (Barc), 2001. **37**(2): p. 85-96.
4. Vermeire, S., G. Van Assche, and P. Rutgeerts, *Laboratory markers in IBD: useful, magic, or unnecessary toys?* Gut, 2006. **55**(3): p. 426-31.
5. Kane, S.V., et al., *Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation.* Am J Gastroenterol, 2003. **98**(6): p. 1309-14.
6. Sugi, K., et al., *Fecal lactoferrin as a marker for disease activity in inflammatory bowel disease: comparison with other neutrophil-derived proteins.* Am J Gastroenterol, 1996. **91**(5): p. 927-34.
7. Vermeire, S., G. Van Assche, and P. Rutgeerts, *C-reactive protein as a marker for inflammatory bowel disease.* Inflamm Bowel Dis, 2004. **10**(5): p. 661-5.
8. Vermeire, S., G. Van Assche, and P. Rutgeerts, *The role of C-reactive protein as an inflammatory marker in gastrointestinal diseases.* Nat Clin Pract Gastroenterol Hepatol, 2005. **2**(12): p. 580-6.
9. Janowitz, H.D., *Burrill B. Crohn (1884-1983).* Mt Sinai J Med, 2000. **67**(1): p. 12-3.
10. Crohn, B.B., L. Ginzburg, and G.D. Oppenheimer, *Regional ileitis; a pathologic and clinical entity.* Am J Med, 1952. **13**(5): p. 583-90.
11. Loftus, E.V., Jr., *Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences.* Gastroenterology, 2004. **126**(6): p. 1504-17.
12. Shivananda, S., et al., *Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD).* Gut, 1996. **39**(5): p. 690-7.
13. Lapidus, A., et al., *Incidence of Crohn's disease in Stockholm County 1955-1989.* Gut, 1997. **41**(4): p. 480-6.
14. Rubin, G.P., et al., *Inflammatory bowel disease: epidemiology and management in an English general practice population.* Aliment Pharmacol Ther, 2000. **14**(12): p. 1553-9.
15. Ardizzone, S. and G. Bianchi Porro, *Inflammatory bowel disease: new insights into pathogenesis and treatment.* J Intern Med, 2002. **252**(6): p. 475-96.

16. *Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls.* Nature, 2007. **447**(7145): p. 661-78.
17. Lees, C.W. and J. Satsangi, *Genetics of inflammatory bowel disease: implications for disease pathogenesis and natural history.* Expert Rev Gastroenterol Hepatol, 2009. **3**(5): p. 513-34.
18. Chamberlin, W., et al., *Review article: Mycobacterium avium subsp. paratuberculosis as one cause of Crohn's disease.* Aliment Pharmacol Ther, 2001. **15**(3): p. 337-46.
19. MacIntyre, C.R. and P.B. McIntyre, *MMR, autism and inflammatory bowel disease: responding to patient concerns using an evidence-based framework.* Med J Aust, 2001. **175**(3): p. 127-8.
20. Matzinger, P., *An innate sense of danger.* Ann N Y Acad Sci, 2002. **961**: p. 341-2.
21. Fiocchi, C., *Inflammatory bowel disease: etiology and pathogenesis.* Gastroenterology, 1998. **115**(1): p. 182-205.
22. Hendrickson, B.A., R. Gokhale, and J.H. Cho, *Clinical aspects and pathophysiology of inflammatory bowel disease.* Clin Microbiol Rev, 2002. **15**(1): p. 79-94.
23. Miller, E., D. Goldblatt, and F. Cutts, *Measles vaccination and inflammatory bowel disease.* Lancet, 1998. **351**(9104): p. 755-6.
24. Wakefield, A.J., et al., *Evidence of persistent measles virus infection in Crohn's disease.* J Med Virol, 1993. **39**(4): p. 345-53.
25. Harries, A.D., et al., *Smoking habits and inflammatory bowel disease: effect on nutrition.* Br Med J (Clin Res Ed), 1982. **284**(6323): p. 1161.
26. Sutherland, L.R., et al., *Effect of cigarette smoking on recurrence of Crohn's disease.* Gastroenterology, 1990. **98**(5 Pt 1): p. 1123-8.
27. Cottone, M., et al., *Smoking habits and recurrence in Crohn's disease.* Gastroenterology, 1994. **106**(3): p. 643-8.
28. Cosnes, J., et al., *Smoking cessation and the course of Crohn's disease: an intervention study.* Gastroenterology, 2001. **120**(5): p. 1093-9.
29. Lindberg, E., G. Jarnerot, and B. Huitfeldt, *Smoking in Crohn's disease: effect on localisation and clinical course.* Gut, 1992. **33**(6): p. 779-82.
30. Timmer, A., L.R. Sutherland, and F. Martin, *Oral contraceptive use and smoking are risk factors for relapse in Crohn's disease. The Canadian Mesalamine for Remission of Crohn's Disease Study Group.* Gastroenterology, 1998. **114**(6): p. 1143-50.
31. Yamamoto, T. and M.R. Keighley, *The association of cigarette smoking with a high risk of recurrence after ileocolonic resection for ileocecal Crohn's disease.* Surg Today, 1999. **29**(6): p. 579-80.
32. Yamamoto, T., R.N. Allan, and M.R. Keighley, *Smoking is a predictive factor for outcome after colectomy and ileorectal anastomosis in patients with Crohn's colitis.* Br J Surg, 1999. **86**(8): p. 1069-70.

33. Ryan, W.R., et al., *Crohn's disease patients who quit smoking have a reduced risk of reoperation for recurrence.* Am J Surg, 2004. **187**(2): p. 219-25.
34. Martin, G., F. Heyen, and S. Dube, *[Factors of recurrence in Crohn disease].* Ann Chir, 1994. **48**(8): p. 685-90.
35. Medina, C., et al., *Influence of the smoking habit in the surgery of inflammatory bowel disease.* Rev Esp Enferm Dig, 1998. **90**(11): p. 771-8.
36. Ryan, W.R., et al., *Patients with Crohn's disease are unaware of the risks that smoking has on their disease.* J Gastrointest Surg, 2003. **7**(5): p. 706-11.
37. Shields, P.L. and T.S. Low-Beer, *Patients' awareness of adverse relation between Crohn's disease and their smoking: questionnaire survey.* BMJ, 1996. **313**(7052): p. 265-6.
38. Yamamoto, T. and M.R. Keighley, *Smoking and disease recurrence after operation for Crohn's disease.* Br J Surg, 2000. **87**(4): p. 398-404.
39. Cosnes, J., *Tobacco and IBD: relevance in the understanding of disease mechanisms and clinical practice.* Best Pract Res Clin Gastroenterol, 2004. **18**(3): p. 481-96.
40. Hauser, W. and D. Grandt, *[Tobacco associated gastrointestinal disorders: smoking cessation therapy - a task for gastroenterologists].* Z Gastroenterol, 2002. **40**(9): p. 815-21.
41. Tobin, M.V., et al., *Cigarette smoking and inflammatory bowel disease.* Gastroenterology, 1987. **93**(2): p. 316-21.
42. Cosnes, J., et al., *Effects of cigarette smoking on the long-term course of Crohn's disease.* Gastroenterology, 1996. **110**(2): p. 424-31.
43. Lindberg, E., et al., *Smoking and inflammatory bowel disease. A case control study.* Gut, 1988. **29**(3): p. 352-7.
44. Thomas, G.A., J. Rhodes, and J.R. Ingram, *Mechanisms of disease: nicotine--a review of its actions in the context of gastrointestinal disease.* Nat Clin Pract Gastroenterol Hepatol, 2005. **2**(11): p. 536-44.
45. Munkholm, P., et al., *[Increased incidence of Crohn disease in the county of Copenhagen].* Ugeskr Laeger, 1993. **155**(40): p. 3199-202.
46. Card, T., R. Hubbard, and R.F. Logan, *Mortality in inflammatory bowel disease: a population-based cohort study.* Gastroenterology, 2003. **125**(6): p. 1583-90.
47. Carter, M.J., A.J. Lobo, and S.P. Travis, *Guidelines for the management of inflammatory bowel disease in adults.* Gut, 2004. **53 Suppl 5**: p. V1-16.
48. Hanauer, S.B. and U. Stromberg, *Oral Pentasa in the treatment of active Crohn's disease: A meta-analysis of double-blind, placebo-controlled trials.* Clin Gastroenterol Hepatol, 2004. **2**(5): p. 379-88.

49. Prantera, C., et al., *Mesalamine in the treatment of mild to moderate active Crohn's ileitis: results of a randomized, multicenter trial.* Gastroenterology, 1999. **116**(3): p. 521-6.
50. Summers, R.W., et al., *National Cooperative Crohn's Disease Study: results of drug treatment.* Gastroenterology, 1979. **77**(4 Pt 2): p. 847-69.
51. Feagan, B.G., *5-ASA therapy for active Crohn's disease: old friends, old data, and a new conclusion.* Clin Gastroenterol Hepatol, 2004. **2**(5): p. 376-8.
52. Modigliani, R., et al., *Clinical, biological, and endoscopic picture of attacks of Crohn's disease. Evolution on prednisolone. Groupe d'Etude Therapeutique des Affections Inflammatoires Digestives.* Gastroenterology, 1990. **98**(4): p. 811-8.
53. Malchow, H., et al., *European Cooperative Crohn's Disease Study (ECCDS): results of drug treatment.* Gastroenterology, 1984. **86**(2): p. 249-66.
54. Tiede, I., et al., *CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes.* J Clin Invest, 2003. **111**(8): p. 1133-45.
55. Sandborn, W., et al., *Azathioprine or 6-mercaptopurine for inducing remission of Crohn's disease.* Cochrane Database Syst Rev, 2000(2): p. CD000545.
56. Fraser, A.G., *Methotrexate: first-line or second-line immunomodulator?* Eur J Gastroenterol Hepatol, 2003. **15**(3): p. 225-31.
57. Rutgeerts, P., G. Van Assche, and S. Vermeire, *Optimizing anti-TNF treatment in inflammatory bowel disease.* Gastroenterology, 2004. **126**(6): p. 1593-610.
58. Hanauer, S.B., et al., *Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial.* Lancet, 2002. **359**(9317): p. 1541-9.
59. Greenstein, A.J., et al., *Perforating and non-perforating indications for repeated operations in Crohn's disease: evidence for two clinical forms.* Gut, 1988. **29**(5): p. 588-92.
60. Andrews, H.A., et al., *Strategy for management of distal ileal Crohn's disease.* Br J Surg, 1991. **78**(6): p. 679-82.
61. Scott, H.J. and J.M. Northover, *Evaluation of surgery for perianal Crohn's fistulas.* Dis Colon Rectum, 1996. **39**(9): p. 1039-43.
62. Yamamoto, T., et al., *Gastroduodenal fistulas in Crohn's disease: clinical features and management.* Dis Colon Rectum, 1998. **41**(10): p. 1287-92.
63. Dietz, D.W., et al., *Safety and longterm efficacy of strictureplasty in 314 patients with obstructing small bowel Crohn's disease.* J Am Coll Surg, 2001. **192**(3): p. 330-7; discussion 337-8.
64. Bernell, O., A. Lapidus, and G. Hellers, *Risk factors for surgery and recurrence in 907 patients with primary ileocaecal Crohn's disease.* Br J Surg, 2000. **87**(12): p. 1697-701.

65. Heimann, T.M., et al., *Comparison of primary and reoperative surgery in patients with Crohns disease.* Ann Surg, 1998. **227**(4): p. 492-5.
66. Kim, N.K., et al., *Long-term outcome after ileocecal resection for Crohn's disease.* Am Surg, 1997. **63**(7): p. 627-33.
67. Yamamoto, T., *Factors affecting recurrence after surgery for Crohn's disease.* World J Gastroenterol, 2005. **11**(26): p. 3971-9.
68. Raab, Y., et al., *Factors influencing recurrence in Crohn's disease. An analysis of a consecutive series of 353 patients treated with primary surgery.* Dis Colon Rectum, 1996. **39**(8): p. 918-25.
69. Anseline, P.F., J. Wlodarczyk, and R. Murugasu, *Presence of granulomas is associated with recurrence after surgery for Crohn's disease: experience of a surgical unit.* Br J Surg, 1997. **84**(1): p. 78-82.
70. Lautenbach, E., J.A. Berlin, and G.R. Lichtenstein, *Risk factors for early postoperative recurrence of Crohn's disease.* Gastroenterology, 1998. **115**(2): p. 259-67.
71. Wolff, B.G., *Factors determining recurrence following surgery for Crohn's disease.* World J Surg, 1998. **22**(4): p. 364-9.
72. Borley, N.R., N.J. Mortensen, and D.P. Jewell, *Preventing postoperative recurrence of Crohn's disease.* Br J Surg, 1997. **84**(11): p. 1493-502.
73. Mekhjian, H.S., et al., *National Cooperative Crohn's Disease Study: factors determining recurrence of Crohn's disease after surgery.* Gastroenterology, 1979. **77**(4 Pt 2): p. 907-13.
74. Whelan, G., et al., *Recurrence after surgery in Crohn's disease. Relationship to location of disease (clinical pattern) and surgical indication.* Gastroenterology, 1985. **88**(6): p. 1826-33.
75. Michelassi, F., et al., *Primary and recurrent Crohn's disease. Experience with 1379 patients.* Ann Surg, 1991. **214**(3): p. 230-8; discussion 238-40.
76. Rutgeerts, P., et al., *Natural history of recurrent Crohn's disease at the ileocolonic anastomosis after curative surgery.* Gut, 1984. **25**(6): p. 665-72.
77. Rutgeerts, P., et al., *Predictability of the postoperative course of Crohn's disease.* Gastroenterology, 1990. **99**(4): p. 956-63.
78. Agrez, M.V., et al., *Surgical history of Crohn's disease in a well-defined population.* Mayo Clin Proc, 1982. **57**(12): p. 747-52.
79. Shivananda, S., et al., *Crohn's disease: risk of recurrence and reoperation in a defined population.* Gut, 1989. **30**(7): p. 990-5.
80. Andrews, H.A., P. Lewis, and R.N. Allan, *Prognosis after surgery for colonic Crohn's disease.* Br J Surg, 1989. **76**(11): p. 1184-90.
81. D'Haens, G.R., A.E. Gasparaitis, and S.B. Hanauer, *Duration of recurrent ileitis after ileocolonic resection correlates with presurgical extent of Crohn's disease.* Gut, 1995. **36**(5): p. 715-7.

82. Heimann, T.M., et al., *Prediction of early symptomatic recurrence after intestinal resection in Crohn's disease.* Ann Surg, 1993. **218**(3): p. 294-8; discussion 298-9.
83. Bernell, O., A. Lapidus, and G. Hellers, *Risk factors for surgery and postoperative recurrence in Crohn's disease.* Ann Surg, 2000. **231**(1): p. 38-45.
84. Sachar, D.B., et al., *Risk factors for postoperative recurrence of Crohn's disease.* Gastroenterology, 1983. **85**(4): p. 917-21.
85. Aeberhard, P., et al., *Surgical recurrence of perforating and nonperforating Crohn's disease. A study of 101 surgically treated Patients.* Dis Colon Rectum, 1996. **39**(1): p. 80-7.
86. Yamamoto, T., R.N. Allan, and M.R. Keighley, *Perforating ileocecal Crohn's disease does not carry a high risk of recurrence but usually re-presents as perforating disease.* Dis Colon Rectum, 1999. **42**(4): p. 519-24.
87. Welsch, T., et al., *Early re-laparotomy for post-operative complications is a significant risk factor for recurrence after ileocaecal resection for Crohn's disease.* Int J Colorectal Dis, 2007. **22**(9): p. 1043-9.
88. Fazio, V.W., et al., *Effect of resection margins on the recurrence of Crohn's disease in the small bowel. A randomized controlled trial.* Ann Surg, 1996. **224**(4): p. 563-71; discussion 571-3.
89. Martel, P., et al., *Crohn's colitis: experience with segmental resections; results in a series of 84 patients.* J Am Coll Surg, 2002. **194**(4): p. 448-53.
90. Speranza, V., et al., *Recurrence of Crohn's disease after resection. Are there any risk factors?* J Clin Gastroenterol, 1986. **8**(6): p. 640-6.
91. Yamamoto, T. and M.R. Keighley, *Long-term results of strictureplasty without synchronous resection for jejunoileal Crohn's disease.* Scand J Gastroenterol, 1999. **34**(2): p. 180-4.
92. Spencer, M.P., et al., *Strictureplasty for obstructive Crohn's disease: the Mayo experience.* Mayo Clin Proc, 1994. **69**(1): p. 33-6.
93. Chung, R.S., *Blood flow in colonic anastomoses. Effect of stapling and suturing.* Ann Surg, 1987. **206**(3): p. 335-9.
94. Moskovitz, D., et al., *Operative and environmental risk factors for recurrence of Crohn's disease.* Int J Colorectal Dis, 1999. **14**(4-5): p. 224-6.
95. Kusunoki, M., et al., *A comparison of stapled and hand-sewn anastomoses in Crohn's disease.* Dig Surg, 1998. **15**(6): p. 679-82.
96. Kotanagi, H., et al., *Do microscopic abnormalities at resection margins correlate with increased anastomotic recurrence in Crohn's disease? Retrospective analysis of 100 cases.* Dis Colon Rectum, 1991. **34**(10): p. 909-16.
97. Viscido, A., et al., *"Crohn's disease activity index" is inaccurate to detect the post-operative recurrence in Crohn's disease. A GISC study. Gruppo Italiano per lo Studio del Colon e del Retto.* Ital J Gastroenterol Hepatol, 1999. **31**(4): p. 274-9.

98. McLeod, R.S., et al., *Risk and significance of endoscopic/radiological evidence of recurrent Crohn's disease.* Gastroenterology, 1997. **113**(6): p. 1823-7.
99. Holzheimer, R.G., R.G. Molloy, and D.H. Wittmann, *Postoperative complications predict recurrence of Crohn's disease.* Eur J Surg, 1995. **161**(2): p. 129-35.
100. Scarpa, M., et al., *Role of stapled and hand-sewn anastomoses in recurrence of Crohn's disease.* Hepatogastroenterology, 2004. **51**(58): p. 1053-7.
101. Poggioli, G., et al., *Factors affecting recurrence in Crohn's disease. Results of a prospective audit.* Int J Colorectal Dis, 1996. **11**(6): p. 294-8.
102. Achkar, J.P. and S.B. Hanauer, *Medical therapy to reduce postoperative Crohn's disease recurrence.* Am J Gastroenterol, 2000. **95**(5): p. 1139-46.
103. Camma, C., et al., *Mesalamine in the maintenance treatment of Crohn's disease: a meta-analysis adjusted for confounding variables.* Gastroenterology, 1997. **113**(5): p. 1465-73.
104. Rutgeerts, P., et al., *Controlled trial of metronidazole treatment for prevention of Crohn's recurrence after ileal resection.* Gastroenterology, 1995. **108**(6): p. 1617-21.
105. Rutgeerts, P., et al., *Ornidazole for prophylaxis of postoperative Crohn's disease recurrence: a randomized, double-blind, placebo-controlled trial.* Gastroenterology, 2005. **128**(4): p. 856-61.
106. D'Haens, G., K. Geboes, and P. Rutgeerts, *Endoscopic and histologic healing of Crohn's (ileo-) colitis with azathioprine.* Gastrointest Endosc, 1999. **50**(5): p. 667-71.
107. D'Haens, G., et al., *Healing of severe recurrent ileitis with azathioprine therapy in patients with Crohn's disease.* Gastroenterology, 1997. **112**(5): p. 1475-81.
108. Hellers, G., et al., *Oral budesonide for prevention of postsurgical recurrence in Crohn's disease. The IOIBD Budesonide Study Group.* Gastroenterology, 1999. **116**(2): p. 294-300.
109. Rutgeerts, P., *Strategies in the prevention of post-operative recurrence in Crohn's disease.* Best Pract Res Clin Gastroenterol, 2003. **17**(1): p. 63-73.
110. Poullis, A., et al., *Review article: faecal markers in the assessment of activity in inflammatory bowel disease.* Aliment Pharmacol Ther, 2002. **16**(4): p. 675-81.
111. Fagerhol, M.K., I. Dale, and T. Andersson, *A radioimmunoassay for a granulocyte protein as a marker in studies on the turnover of such cells.* Bull Eur Physiopathol Respir, 1980. **16 Suppl**: p. 273-82.
112. Tibble, J., et al., *A simple method for assessing intestinal inflammation in Crohn's disease.* Gut, 2000. **47**(4): p. 506-13.
113. Saverymuttu, S.H., et al., *111Indium autologous leucocytes in inflammatory bowel disease.* Gut, 1983. **24**(4): p. 293-9.

114. Saverymuttu, S.H., et al., *Assessment of disease activity in ulcerative colitis using indium-111-labelled leukocyte faecal excretion.* Scand J Gastroenterol, 1983. **18**(7): p. 907-12.
115. Gaya, D.R., et al., *Faecal calprotectin in the assessment of Crohn's disease activity.* QJM, 2005. **98**(6): p. 435-41.
116. Isaksen, B. and M.K. Fagerhol, *Calprotectin inhibits matrix metalloproteinases by sequestration of zinc.* Mol Pathol, 2001. **54**(5): p. 289-92.
117. Yui, S., M. Mikami, and M. Yamazaki, *Induction of apoptotic cell death in mouse lymphoma and human leukemia cell lines by a calcium-binding protein complex, calprotectin, derived from inflammatory peritoneal exudate cells.* J Leukoc Biol, 1995. **58**(6): p. 650-8.
118. Roseth, A.G., et al., *Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study.* Scand J Gastroenterol, 1992. **27**(9): p. 793-8.
119. Bunn, S.K., et al., *Fecal calprotectin: validation as a noninvasive measure of bowel inflammation in childhood inflammatory bowel disease.* J Pediatr Gastroenterol Nutr, 2001. **33**(1): p. 14-22.
120. Fagerberg, U.L., et al., *Fecal calprotectin: a quantitative marker of colonic inflammation in children with inflammatory bowel disease.* J Pediatr Gastroenterol Nutr, 2007. **45**(4): p. 414-20.
121. Fagerberg, U.L., et al., *Fecal calprotectin levels in healthy children studied with an improved assay.* J Pediatr Gastroenterol Nutr, 2003. **37**(4): p. 468-72.
122. Lundberg, J.O., et al., *Technology insight: calprotectin, lactoferrin and nitric oxide as novel markers of inflammatory bowel disease.* Nat Clin Pract Gastroenterol Hepatol, 2005. **2**(2): p. 96-102.
123. Meling, T.R., et al., *Faecal calprotectin shedding after short-term treatment with non-steroidal anti-inflammatory drugs.* Scand J Gastroenterol, 1996. **31**(4): p. 339-44.
124. Tibble, J.A., et al., *High prevalence of NSAID enteropathy as shown by a simple faecal test.* Gut, 1999. **45**(3): p. 362-6.
125. Poullis, A., et al., *Proton pump inhibitors are associated with elevation of faecal calprotectin and may affect specificity.* Eur J Gastroenterol Hepatol, 2003. **15**(5): p. 573-4; author reply 574.
126. Poullis, A., et al., *Bowel inflammation as measured by fecal calprotectin: a link between lifestyle factors and colorectal cancer risk.* Cancer Epidemiol Biomarkers Prev, 2004. **13**(2): p. 279-84.
127. Carroccio, A., et al., *Diagnostic accuracy of fecal calprotectin assay in distinguishing organic causes of chronic diarrhea from irritable bowel syndrome: a prospective study in adults and children.* Clin Chem, 2003. **49**(6 Pt 1): p. 861-7.
128. Canani, R.B., et al., *Combined use of noninvasive tests is useful in the initial diagnostic approach to a child with suspected inflammatory bowel disease.* J Pediatr Gastroenterol Nutr, 2006. **42**(1): p. 9-15.

129. Costa, F., et al., *Role of faecal calprotectin as non-invasive marker of intestinal inflammation.* Dig Liver Dis, 2003. **35**(9): p. 642-7.
130. D'Inca, R., et al., *Calprotectin and lactoferrin in the assessment of intestinal inflammation and organic disease.* Int J Colorectal Dis, 2007. **22**(4): p. 429-37.
131. Gisbert, J.P. and A.G. McNicholl, *Questions and answers on the role of faecal calprotectin as a biological marker in inflammatory bowel disease.* Dig Liver Dis, 2009. **41**(1): p. 56-66.
132. Limburg, P.J., et al., *Fecal calprotectin levels predict colorectal inflammation among patients with chronic diarrhea referred for colonoscopy.* Am J Gastroenterol, 2000. **95**(10): p. 2831-7.
133. Bremner, A., et al., *Faecal calprotectin in children with chronic gastrointestinal symptoms.* Acta Paediatr, 2005. **94**(12): p. 1855-8.
134. Bunn, S.K., et al., *Fecal calprotectin as a measure of disease activity in childhood inflammatory bowel disease.* J Pediatr Gastroenterol Nutr, 2001. **32**(2): p. 171-7.
135. Kaiser, T., et al., *Faecal S100A12 as a non-invasive marker distinguishing inflammatory bowel disease from irritable bowel syndrome.* Gut, 2007. **56**(12): p. 1706-13.
136. Thjodleifsson, B., et al., *Subclinical intestinal inflammation: an inherited abnormality in Crohn's disease relatives?* Gastroenterology, 2003. **124**(7): p. 1728-37.
137. Schroder, O., et al., *Prospective evaluation of faecal neutrophil-derived proteins in identifying intestinal inflammation: combination of parameters does not improve diagnostic accuracy of calprotectin.* Aliment Pharmacol Ther, 2007. **26**(7): p. 1035-42.
138. Silberer, H., et al., *Fecal leukocyte proteins in inflammatory bowel disease and irritable bowel syndrome.* Clin Lab, 2005. **51**(3-4): p. 117-26.
139. von Roon, A.C., et al., *Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy.* Am J Gastroenterol, 2007. **102**(4): p. 803-13.
140. Fagerberg, U.L., et al., *Colorectal inflammation is well predicted by fecal calprotectin in children with gastrointestinal symptoms.* J Pediatr Gastroenterol Nutr, 2005. **40**(4): p. 450-5.
141. Tibble, J., et al., *Faecal calprotectin and faecal occult blood tests in the diagnosis of colorectal carcinoma and adenoma.* Gut, 2001. **49**(3): p. 402-8.
142. Summerton, C.B., et al., *Faecal calprotectin: a marker of inflammation throughout the intestinal tract.* Eur J Gastroenterol Hepatol, 2002. **14**(8): p. 841-5.
143. Dolwani, S., et al., *Diagnostic accuracy of faecal calprotectin estimation in prediction of abnormal small bowel radiology.* Aliment Pharmacol Ther, 2004. **20**(6): p. 615-21.
144. Langhorst, J., et al., *Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance*

of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. Am J Gastroenterol, 2008. **103**(1): p. 162-9.
145. Tibble, J.A., et al., *Use of surrogate markers of inflammation and Rome criteria to distinguish organic from nonorganic intestinal disease.* Gastroenterology, 2002. **123**(2): p. 450-60.
146. Roseth, A.G., *Determination of faecal calprotectin, a novel marker of organic gastrointestinal disorders.* Dig Liver Dis, 2003. **35**(9): p. 607-9.
147. Gaya, D.R. and J.F. Mackenzie, *Faecal calprotectin: a bright future for assessing disease activity in Crohn's disease.* QJM, 2002. **95**(9): p. 557-8.
148. Langhorst, J., et al., *Comparison of 4 neutrophil-derived proteins in feces as indicators of disease activity in ulcerative colitis.* Inflamm Bowel Dis, 2005. **11**(12): p. 1085-91.
149. Roseth, A.G., et al., *Assessment of disease activity in ulcerative colitis by faecal calprotectin, a novel granulocyte marker protein.* Digestion, 1997. **58**(2): p. 176-80.
150. Roseth, A.G., E. Aadland, and K. Grzyb, *Normalization of faecal calprotectin: a predictor of mucosal healing in patients with inflammatory bowel disease.* Scand J Gastroenterol, 2004. **39**(10): p. 1017-20.
151. Kolho, K.L., et al., *Fecal calprotectin remains high during glucocorticoid therapy in children with inflammatory bowel disease.* Scand J Gastroenterol, 2006. **41**(6): p. 720-5.
152. Casellas, F., et al., *Fecal excretion of deoxyribonucleic acid in long-term follow-up of patients with inactive ulcerative colitis.* Inflamm Bowel Dis, 2007. **13**(4): p. 386-90.
153. Tibble, J.A., et al., *Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease.* Gastroenterology, 2000. **119**(1): p. 15-22.
154. Costa, F., et al., *Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease.* Gut, 2005. **54**(3): p. 364-8.
155. Scarpa, M., et al., *Fecal lactoferrin and calprotectin after ileocolonic resection for Crohn's disease.* Dis Colon Rectum, 2007. **50**(6): p. 861-9.
156. Scarpa, M., et al., *The role of costimulatory molecules CD80 and CD86 and IFNgamma in the pathogenesis of ulcerative colitis.* Dig Dis Sci, 2004. **49**(11-12): p. 1738-44.
157. Aadland, E. and M.K. Fagerhol, *Faecal calprotectin: a marker of inflammation throughout the intestinal tract.* Eur J Gastroenterol Hepatol, 2002. **14**(8): p. 823-5.
158. Orlando, A., et al., *The role of calprotectin in predicting endoscopic post-surgical recurrence in asymptomatic Crohn's disease: a comparison with ultrasound.* Eur Rev Med Pharmacol Sci, 2006. **10**(1): p. 17-22.

159. Masson, P.L., J.F. Heremans, and E. Schonne, *Lactoferrin, an iron-binding protein in neutrophilic leukocytes.* J Exp Med, 1969. **130**(3): p. 643-58.
160. Parsi, M.A., et al., *Ascitic fluid lactoferrin for diagnosis of spontaneous bacterial peritonitis.* Gastroenterology, 2008. **135**(3): p. 803-7.
161. Guerrant, R.L., et al., *Measurement of fecal lactoferrin as a marker of fecal leukocytes.* J Clin Microbiol, 1992. **30**(5): p. 1238-42.
162. Martins, C.A., et al., *Correlation of lactoferrin with neutrophilic inflammation in body fluids.* Clin Diagn Lab Immunol, 1995. **2**(6): p. 763-5.
163. Saitoh, O., et al., *Comparison of tests for fecal lactoferrin and fecal occult blood for colorectal diseases: a prospective pilot study.* Intern Med, 2000. **39**(10): p. 778-82.
164. Uchida, K., et al., *Immunochemical detection of human lactoferrin in feces as a new marker for inflammatory gastrointestinal disorders and colon cancer.* Clin Biochem, 1994. **27**(4): p. 259-64.
165. Desai, D., W.A. Faubion, and W.J. Sandborn, *Review article: biological activity markers in inflammatory bowel disease.* Aliment Pharmacol Ther, 2007. **25**(3): p. 247-55.
166. Gisbert, J.P., Y. Gonzalez-Lama, and J. Mate, *[Role of biological markers in inflammatory bowel disease].* Gastroenterol Hepatol, 2007. **30**(3): p. 117-29.
167. Schoepfer, A.M., et al., *Accuracy of four fecal assays in the diagnosis of colitis.* Dis Colon Rectum, 2007. **50**(10): p. 1697-706.
168. Walker, T.R., et al., *Fecal lactoferrin is a sensitive and specific marker of disease activity in children and young adults with inflammatory bowel disease.* J Pediatr Gastroenterol Nutr, 2007. **44**(4): p. 414-22.
169. Fine, K.D., et al., *Utility of a rapid fecal latex agglutination test detecting the neutrophil protein, lactoferrin, for diagnosing inflammatory causes of chronic diarrhea.* Am J Gastroenterol, 1998. **93**(8): p. 1300-5.
170. Hirata, I., et al., *Usefulness of fecal lactoferrin and hemoglobin in diagnosis of colorectal diseases.* World J Gastroenterol, 2007. **13**(10): p. 1569-74.
171. Dai, J., et al., *Relationship between fecal lactoferrin and inflammatory bowel disease.* Scand J Gastroenterol, 2007. **42**(12): p. 1440-4.
172. Sipponen, T., et al., *Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings.* Inflamm Bowel Dis, 2008. **14**(1): p. 40-6.
173. Gisbert, J.P., A.G. McNicholl, and F. Gomollon, *Questions and answers on the role of fecal lactoferrin as a biological marker in inflammatory bowel disease.* Inflamm Bowel Dis, 2009. **15**(11): p. 1746-54.

174. Schoepfer, A.M., et al., *Discriminating IBD from IBS: comparison of the test performance of fecal markers, blood leukocytes, CRP, and IBD antibodies.* Inflamm Bowel Dis, 2008. **14**(1): p. 32-9.
175. Xiang, J.Y., Q. Ouyang, and G.D. Li, *[Significance of fecal lactoferrin in evaluation of disease activity in ulcerative colitis].* Zhonghua Yi Xue Za Zhi, 2007. **87**(32): p. 2262-4.
176. Saitoh, O., et al., *Fecal eosinophil granule-derived proteins reflect disease activity in inflammatory bowel disease.* Am J Gastroenterol, 1999. **94**(12): p. 3513-20.
177. Otten, C.M., et al., *Diagnostic performance of rapid tests for detection of fecal calprotectin and lactoferrin and their ability to discriminate inflammatory from irritable bowel syndrome.* Clin Chem Lab Med, 2008. **46**(9): p. 1275-80.
178. Sutherland, A.D., R.B. Gearry, and F.A. Frizelle, *Review of fecal biomarkers in inflammatory bowel disease.* Dis Colon Rectum, 2008. **51**(8): p. 1283-91.
179. Tuccari, G., et al., *Iron-binding proteins in human colorectal adenomas and carcinomas: an immunocytochemical investigation.* Histol Histopathol, 1992. **7**(4): p. 543-7.
180. Loftus, E.V., Jr., *Clinical perspectives in Crohn's disease. Objective measures of disease activity: alternatives to symptom indices.* Rev Gastroenterol Disord, 2007. **7 Suppl 2**: p. S8-S16.
181. Sipponen, T., et al., *Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings.* Aliment Pharmacol Ther, 2008. **28**(10): p. 1221-9.
182. Gisbert, J.P., et al., *Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse.* Inflamm Bowel Dis, 2009. **15**(8): p. 1190-8.
183. Buderus, S., et al., *Fecal lactoferrin: a new parameter to monitor infliximab therapy.* Dig Dis Sci, 2004. **49**(6): p. 1036-9.
184. Sipponen, T., et al., *Fecal calprotectin, lactoferrin, and endoscopic disease activity in monitoring anti-TNF-alpha therapy for Crohn's disease.* Inflamm Bowel Dis, 2008. **14**(10): p. 1392-8.
185. Parsi, M.A., et al., *Fecal lactoferrin for diagnosis of symptomatic patients with ileal pouch-anal anastomosis.* Gastroenterology, 2004. **126**(5): p. 1280-6.
186. Harvey, R.F. and J.M. Bradshaw, *A simple index of Crohn's-disease activity.* Lancet, 1980. **1**(8167): p. 514.
187. Jorgensen, L.G., et al., *How accurate are clinical activity indices for scoring of disease activity in inflammatory bowel disease (IBD)?* Clin Chem Lab Med, 2005. **43**(4): p. 403-11.
188. Yoshida, E.M., *The Crohn's Disease Activity Index, its derivatives and the Inflammatory Bowel Disease Questionnaire: a review of instruments to assess Crohn's disease.* Can J Gastroenterol, 1999. **13**(1): p. 65-73.

189. Best, W.R., et al., *Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study.* Gastroenterology, 1976. **70**(3): p. 439-44.
190. Rutgeerts, P., et al., *Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease.* Gastroenterology, 2004. **126**(2): p. 402-13.

Publications
And Presentations

Publications arising from this thesis:

a) Paper -

1) Faecal calprotectin or lactoferrin can identify postoperative recurrence in Crohn's disease. Lamb CA*, **Mohiuddin MK***, Gicquel J, Neely D, Bergin FG, Hanson JM, Mansfield JC. **Br J Surg**. 2009 Jun; 96(6): 663-74. * Both contributed equally for first author to this paper.

b) Commentaries related to BJS Paper –

1) Paper appreciation in Minerva section of **Br Med Journal** / 30 May 2009/ Volume 338

2) Can fecal calprotectin or lactoferrin identify postoperative recurrence in Crohn's disease? Lamb CA, **Mohiuddin MK**, Gicquel J. Selected Summary by Prof Frank Seibold, MD, Alain M. Schoepfer, MD. Division of Gastroenterology Inselspital, University of Bern, Switzerland. **Inflammatory Bowel Diseases.** Oct 2009; Volume 9999: Issue 9999, Crohn's & Colitis Foundation of America. (DOI) 10.1002/ibd. 21173

c) Oral Presentation related to this project -

Moynihan Prize session (Annual Scientific Meeting- Association of Surgeons of Great Britain and Ireland 18-20th April 2007

1) Post operative Crohn's disease: the role of faecal lactoferrin in detecting clinical relapse after ileocaecal resection. **MK Mohiuddin**, J Gicquel, J Robson, C Todhunter, JM Hanson, JC Mansfield. Br J Surg. 2007 Apr; 94(Suppl 2):2

d) Award - First Prize -

1) Post operative Crohn's disease: the role of faecal lactoferrin & calprotectin in detecting clinical relapse after ileocaecal resection. **MK Mohiuddin**, JM Hanson, JC Mansfield. **Mrs Ella Forster Memorial Award** at Newcastle upon Tyne Hospitals NHS foundation trust, Freeman Hospital, Newcastle 2nd March 2007.

c) Poster presentation -

1) **Mohiuddin MK**, Lamb CA, Gicquel J, Neely D, Bergin FG, Hanson JM, Mansfield JC. Post operative Crohn's disease - The role of faecal calprotectin and lactoferrin in assessing disease recurrence following ileal resection. *Gut* 2009; 58(Suppl I): A58.

2) **Mohiuddin MK**, Lamb CA, Gicquel J, Neely D, Bergin FG, Hanson JM, Mansfield JC. Longitudinal study of faecal markers following ileal resection in Crohn's disease - Do calprotectin and lactoferrin normalise post operatively. *Gut* 2009; 58(Supp I): A58.

3) Lamb CA, **Mohiuddin MK**, Gicquel J, Neely D, Bergin FG, Hanson JM, Mansfield JC. Faecal lactoferrin can identify post-operative disease recurrence, and may allow immunosuppressant targeting following ileal resection in Crohn's disease. *Gastroenterology* 2009; 136(5): A-667.

Original article

Faecal calprotectin or lactoferrin can identify postoperative recurrence in Crohn's disease

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Background: Identifying Crohn's disease recurrence in symptomatic patients after ileocaecal resection is difficult. The aim of this study was to evaluate faecal concentrations of granulocyte degradation products in this setting.

Methods: A postoperative cohort of 13 patients was followed prospectively for 1 year with regular faecal calprotectin (FC) and lactoferrin (FL) measurements. A second postoperative cohort (median 24 months after resection) of 104 patients provided a single stool sample. Faecal measurements were compared with symptom diaries, the Harvey Bradshaw Index, endoscopic examination, C-reactive protein and platelet measurement.

Results: In the uncomplicated course, both markers normalized within 2 months. Both FC and FL correlated significantly with Harvey Bradshaw Index ($P < 0{\cdot}001$). Twenty-eight patients with severely clinically active disease had high mean(s.e.) levels of FC (661·1(119·1) μg/g) and FL (116·6(32·2) μg/g); and 43 with clinically inactive disease had low levels of FC (70·2(27·1) μg/g) and FL (5·9(2·4) μg/g). In patients with mild to moderately clinically active disease, FC and FL identified individuals with and without recurrent inflammatory disease. Faecal markers were more accurate at predicting clinical disease activity than C-reactive protein, platelet count or endoscopic appearance.

Conclusion: FC and FL are non-invasive tests that can help to identify disease recurrence in symptomatic postoperative patients.

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Introduction

Crohn's disease is a chronic inflammatory bowel disease characterized by periods of remission and relapse usually resulting from intestinal inflammation. More than 80 per cent of patients with Crohn's disease require surgery in the first 10 years after disease onset[1]. Within weeks of resection new aphthous ulceration in the neoterminal ileum can be demonstrated in most patients, and by 1 year up to 80 per cent have endoscopic evidence of recurrence[2,3]. By 3 to 5 years after surgery, around a third of patients will have a clinical relapse[1,2]. However, the remainder may experience a far more benign disease course, remaining in long-term remission.

There is poor understanding of the factors that cause, or protect against, postoperative recurrence. Smoking, perforating behaviour of disease, ileal or ileocolonic location and female sex have all been associated with a tendency to early and aggressive recurrence after resection[4–8]. Recognition of these factors can provide a means to stratify risk broadly within a population, but prediction of recurrence in an individual remains poor.

After operative intestinal resection, patients with Crohn's disease often develop symptoms suggestive of disease relapse. Clinically it is hard to establish whether

The Editors are satisfied that all authors have contributed significantly to this publication

these symptoms are due to genuine recurrence of gut inflammation or rapid gut transit as a consequence of altered anatomy, bile salt malabsorption or functional bowel disorder.

Effective treatment of postoperative disease recurrence is possible using immunosuppressant agents such as azathioprine, 6-mercaptopurine and antitumour necrosis factor (TNF) α antibody therapy, all of which can produce mucosal healing[9,10]. However, they do have side-effects and it is therefore desirable to target these therapies to patients with evidence of intestinal inflammation; those without gut inflammation may find symptomatic improvement from antimotility agents such as loperamide or cholestyramine.

In practice, it is difficult to separate symptomatic patients with disease recurrence from those without. Non-invasive clinical indices such as the Crohn's Disease Activity Index (CDAI)[11] or the more simplified Harvey Bradshaw Index (HBI)[12] may be useful. The former is complex and time consuming, involving a detailed patient diary, and is therefore used mainly in clinical trials; although it is reproducible, it was not developed for use after ileocolonic resection and has not been validated in this setting. The CDAI is also poorly predictive in those with fistulating and stenosing disease[13], and is limited by significant observer variability[14]. Laboratory markers, especially C-reactive protein (CRP), may be prognostically relevant in predicting relapse of Crohn's in the long term but not within the first year of resection[15]. Radiological investigation may miss early recurrence and exposes patients to high radiation doses. Endoscopy with biopsy, although considered the 'gold standard' test, is invasive, expensive and labour intensive, and endoscopic inflammation often does not correlate with clinical relapse[16]. Furthermore, in small bowel disease conventional endoscopic evaluation may not allow access to the relevant area of diseased gut, and capsule enteroscopy, which is expensive, has not been used widely in this setting.

The ideal assessment of the postoperative patient would be a reliable, non-invasive marker of recurrent disease that can be repeatedly monitored, correlating both with symptom relapse and mucosal ulceration. Previous research has demonstrated that in Crohn's disease there is migration of inflammatory cells to the diseased gut mucosa[17–19]. Faecal concentrations of granulocyte degradation products may therefore offer a suitable method for assessment of disease activity after ileocolonic resection.

Faecal calprotectin (FC), a granulocyte cytosolic protein, is closely correlated with 5-day faecal excretion of indium-111-labelled leucocytes, which is widely regarded as the 'gold standard' for quantification of intestinal inflammation[17,18,20,21]. It is released during cell death and so is a marker of cell turnover and hence inflammation. It has been established previously that patients with inflammatory bowel disease (IBD) in remission have normal FC concentrations[22–26]. Faecal lactoferrin (FL)[27] is an iron-binding glycoprotein secreted by most mucosal membranes, and is a major component of secondary granules in neutrophil granulocytes, a primary component of an acute inflammatory response[28]. FL levels quickly increase after the influx of neutrophils into the intestinal lumen during inflammation[29], and concentrations are significantly raised in clinically active adult and paediatric IBD[30–32]. These markers are simple to use, and previous research in Crohn's disease has shown them to be sensitive for intestinal inflammation, reliable, inexpensive, non-invasive, and able to differentiate organic from non-organic disease[22,23,30,33–35]. Far less research has investigated the potential use of FC and FL in the postoperative setting.

The hypothesis of the present study was that quantitative measurement of markers FC and FL may offer a valuable, non-invasive method of assessing the symptomatic postoperative patient in Crohn's disease. Specific aims were as follows: to determine the immediate postoperative course of FC and FL after ileocaecal resection, and to determine whether these faecal markers could demonstrate inflammatory activity and identify postoperative disease recurrence; to investigate how FC and FL correlate with each other after resection; to determine whether FC and FL measurements in the symptomatic postoperative patient can differentiate those with aggressive disease from those with indolent or benign disease; and to compare FC and FL with other measures of inflammatory activity such as CRP, platelet count and endoscopic examination.

Methods

The investigation had two related arms: a longitudinal study to monitor the trend of postoperative FC and FL concentrations, and a cross-sectional study to determine whether FC and FL measurement could identify gut inflammation in patients with symptomatic postoperative Crohn's disease. Specific methods for both arms are outlined below.

To be included, patients had to have a histological diagnosis of Crohn's disease and an ileocaecal resection between January 1999 and August 2007. Patients were identified for study through the medical and surgical gastroenterology clinics at the Newcastle upon Tyne Hospitals NHS Foundation Trust.

Table 1 Demographic and clinical profile of cross-sectional and longitudinal study groups

	Cross-sectional ($n = 104$)	Longitudinal ($n = 13$)
Sex ratio (M : F)	43 : 61	4 : 9
Age (years)*	45 (18–79)	34 (18–64)
Duration of disease (years)*	12 (1–40)	8 (1–30)
Time after ileocaecal resection (months)*	24 (2–300)	–
Disease location		
Ileal	71	10
Ileocolonic	28	3
Perianal†	5	1
Disease behaviour		
Inflammatory	17	3
Stenosing	51	7
Perforating	31	3
Perianal fistula†	5	1
Smoking status		
Never	31	5
Former	32	3
Current	41	5
Endoscopy (<1 month from follow-up)		
Recurrence	25 of 43	3 of 4
No recurrence	18 of 43	1 of 4
Maintenance therapy		
No medication	33	5
5-Aminosalicylic acid	10	3
Corticosteroids	23	4
Azathioprine	39	8
Methotrexate	11	2
Antitumour necrosis factor α antibodies	3	2

*Values are median (range). †Perianal disease in combination with ileal or ileocolonic disease.

Overall 140 patients were invited to participate, and 23 were lost at the stage of stool collection. In total 117 patients were studied (*Table 1*).

A control group was not required for this study as the upper limit of normal ranges for these faecal markers are well defined (calprotectin, less than 50 μg/g; lactoferrin, less than 7·25 μg/g).

All patients gave written informed consent before inclusion in the study. Ethical approval for the study was obtained from the Gateshead and South Tyneside local research and ethics committee.

Longitudinal study

A group of 13 patients who had undergone ileocaecal resection for symptomatic Crohn's disease were prospectively followed for 12 months. Data on the postoperative course, complications, the need for further interventions and outcome were collected. Serial stool samples before surgery, at weekly intervals for 4 weeks and monthly intervals for 12 months were collected to determine the trends in FC and FL levels after surgery.

Cross-sectional study

A group of 104 patients who had undergone a previous ileocaecal resection for Crohn's disease with a mean duration of 24 (range 2–300) months since surgery were recruited. The following clinical characteristics were recorded from patient notes and direct interview: age at disease or symptom onset, sex, smoking history, disease behaviour, pharmacological therapy and ileocolonoscopy findings (considered valid if performed within 4 weeks of the patient interview). The HBI was also calculated for each patient in order to estimate clinical disease activity. A score of 3 or less indicated clinically inactive disease; 4, mildly active; 5, moderately active; and 6 or more, severely active disease. Venous blood was obtained for CRP (normal value less than 5 mg/l) and platelet count (normal value $350–500 \times 10^9$/l). On the same day a single stool sample was collected for FC and FL.

Faecal tests

A single stool sample was used to determine the FC and FL levels. The samples were labelled, sealed and stored at −20°C until the assays were performed. Assays were performed in the biochemistry laboratory at Freeman Hospital, Newcastle upon Tyne Hospitals NHS Foundation Trust. The quoted normal ranges for the two markers were confirmed using ten stool specimens obtained from control patients without IBD.

FC was measured using a commercially available quantitative enzyme immunoassay (PhiCal™; Calpro, Lysaker, Norway). FL was measured by quantitative enzyme-linked immunosorbent assay (*IBD-SCAN*®; TechLab, Blacksburg, Virginia, USA).

The techniques for measurement of both faecal markers followed the manufacturers' guidelines and have been successfully used in previous research[30,36,37].

Statistical analysis

Data analysis was undertaken using SPSS® for Windows® version 15.0 (SPSS, Chicago, Illinois, USA). For non-parametric data, the Kruskal–Wallis test was used to identify significant differences in groups of continuous variables. The Spearman's rank order correlation (r) was used to compare FC or FL and continuous parameters.

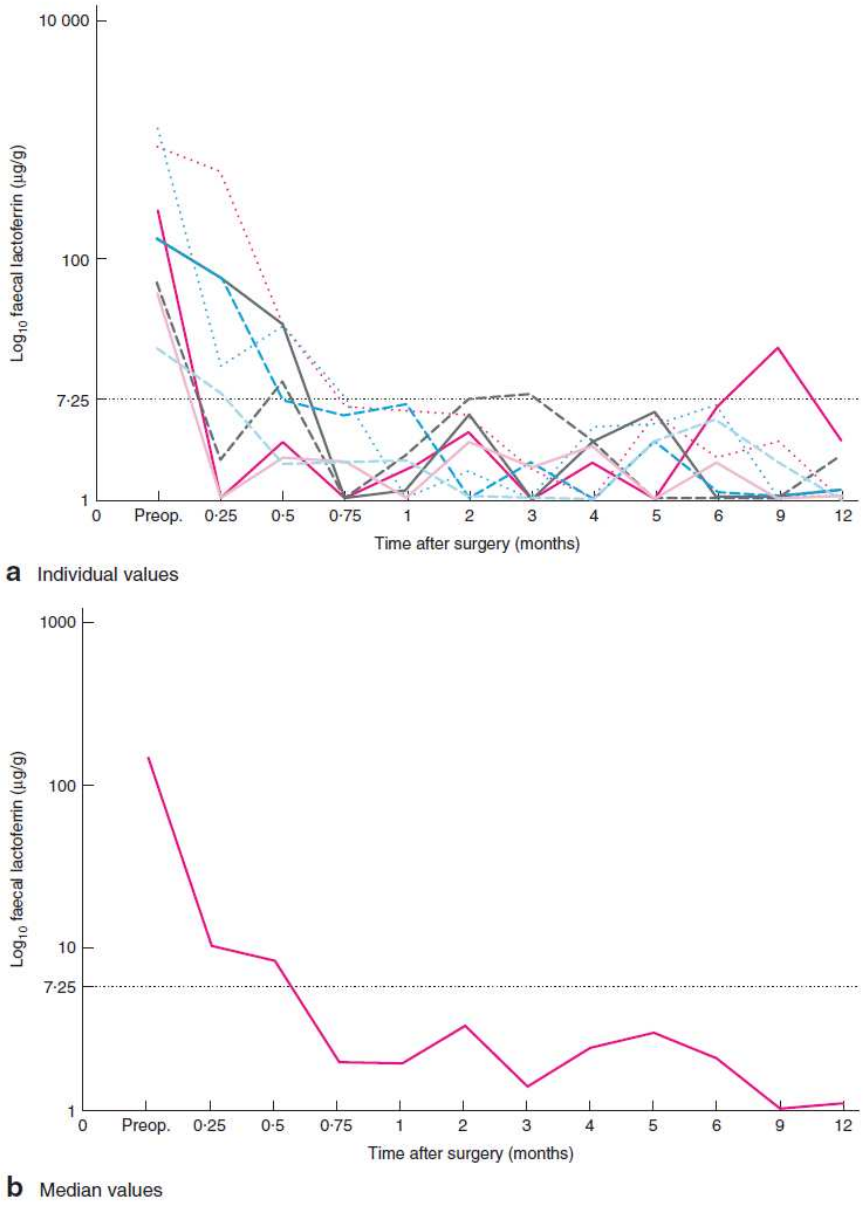

Fig. 1 Faecal lactoferrin concentration before and after surgery for eight patients with an uncomplicated postoperative recovery, **a** showing for the first time that levels in all patients normalize after 2 months, and **b** showing median values. The dotted line represents upper limit of normal

Results

Longitudinal study

This group, set up to determine the course of FC and FL immediately after surgery, included 13 patients. A total of 155 stool samples were collected. Eight patients had an uncomplicated postoperative recovery. FC and FL (mean (s.e.)) were highest before surgery, at 562(155·7) and 347·7(159.0) μg/g respectively, but fell quickly after surgery (*Table 2*). By 2 months, both faecal markers normalized and

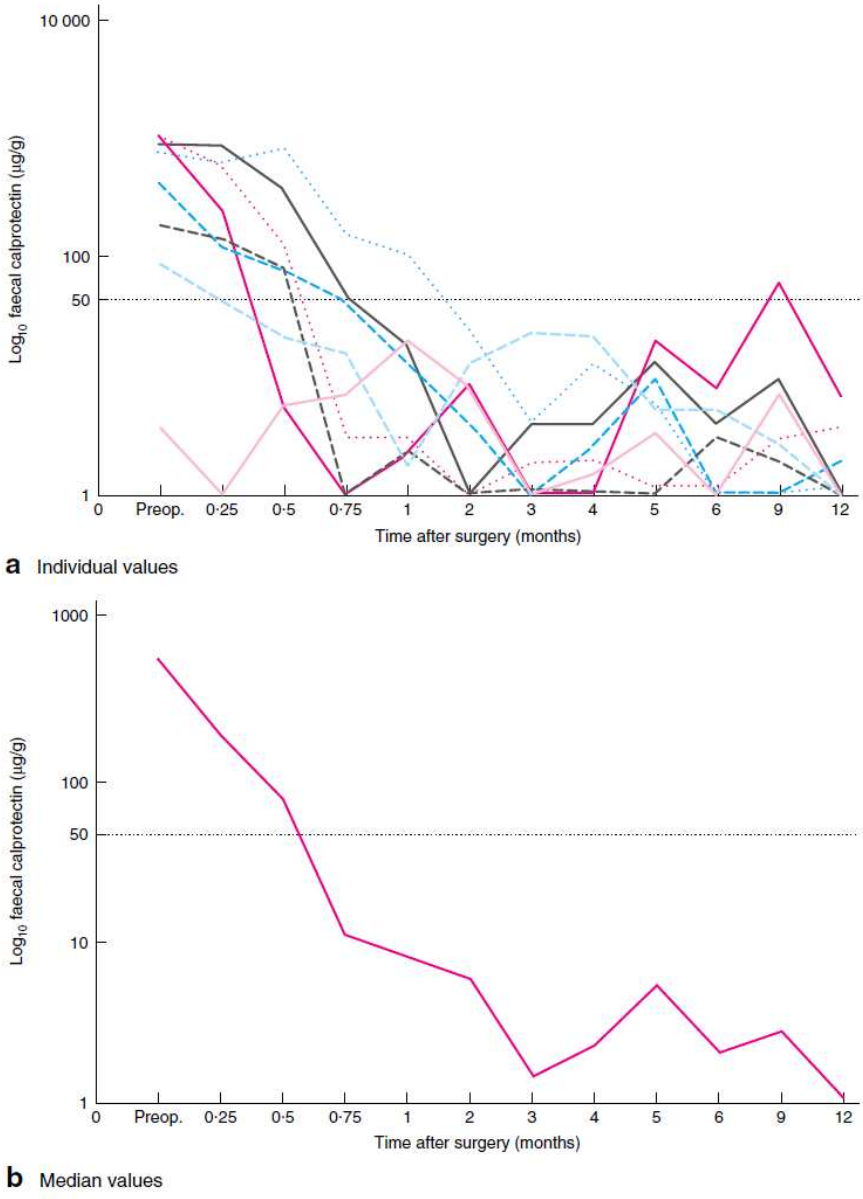

Fig. 2 Faecal calprotectin concentration before surgery and at follow-up for eight patients with an uncomplicated postoperative recovery, showing **a** for the first time that levels in all patients normalize after 2 months, and **b** showing median values. The dotted line represents upper limit of normal

remained within normal limits. In each patient the FC and FL variation with time was similar. The individual trends of FL and FC for each patient are shown in *Figs 1* and *2* respectively.

Five of the 13 patients had complications (*Figs 3* and *4*). Patient 1 was a 58-year-old man who developed an intra-abdominal collection 1 month after ileocaecal resection. His preoperative FL level was 37·9 μg/g, which quickly

Table 2 Faecal marker concentrations before and after surgery for the eight patients with an uncomplicated recovery

	Calprotectin (μg/g)	Lactoferrin (μg/g)
Before surgery	562·0(155·7)	347·7(159·0)
1 week	334·3(114·2)	91·1(68·0)
4 weeks	20·8(12·4)	2·7(0·7)
2 months	8·7(2·8)	3·4(0·7)
3 months	4·7(2·7)	2·1(0·7)
6 months	3·0(0·9)	3·0(0·8)

Values are mean(s.e.).

normalized but rose to 121·5 μg/g by 1 month coinciding with the leak. A preoperative FC level of 519·8 μg/g normalized but then rose by the time of complication to 263·1 μg/g. After repair, both markers normalized within 1 month. Patient 2 was a 69-year-old woman who developed a leak and intra-abdominal collection in the second week after surgery, which was drained at laparotomy and she made a good recovery. At the time of the leak, her FL climbed from 5·8 to 680·8 μg/g and her FC from 30·5 to 1616·0 μg/g. Both normalized within 1 month after drainage of the abscess. Patient 3 was a 25-year-old woman who developed a wound infection as well as clinical and endoscopic evidence of disease recurrence within 1 month of surgery. A preoperative FL level of 179·5 μg/g normalized after surgery to 5·2 μg/g before rising to 116·8 μg/g, when she relapsed. Her preoperative FC level was 1616·8 μg/g. This fell slightly to 1344·5 μg/g and then climbed to 1607·0 μg/g at the time of relapse. After treatment of her wound infection, she was started on azathioprine and both FL and FC normalized over the next 3 months. Patient 4, a 21-year-old man, had preoperative FL and FC concentrations of 61·2 and 1127·4 μg/g respectively. Both normalized by 1 month after surgery. At 9 months, his symptoms flared up again, necessitating azathioprine therapy. At the time of recurrence his normal FL and FC had risen to 22·1 and 78·9 μg/g respectively after consecutive rises from month 5 onwards. Patient 5 was a 34-year-old man with ileocolonic and perianal disease. His preoperative FL and FC were 165·8 and 1707·6 μg/g. Both normalized by the fourth postoperative week. Coinciding with a rise in FC (78·9 μg/g) and FL (22·1 μg/g) at 9 months he relapsed and was started on infliximab. At 12 months, both faecal markers had normalized.

Only one of the patients from the uncomplicated recovery group had an unexplained rise in FL and FC. This patient had high preoperative concentrations of 248·7 and 1035·8 μg/g respectively, which normalized by 2 weeks after surgery. There was a minor rise in both FL and FC at 9 months to 19·1 and 61 μg/g before spontaneous normalization at 1 year.

Cross-sectional study

All FC and FL values for the 104 patients in the cross-sectional study and all 155 stool samples used in the longitudinal study (a total of 259 data points) showed

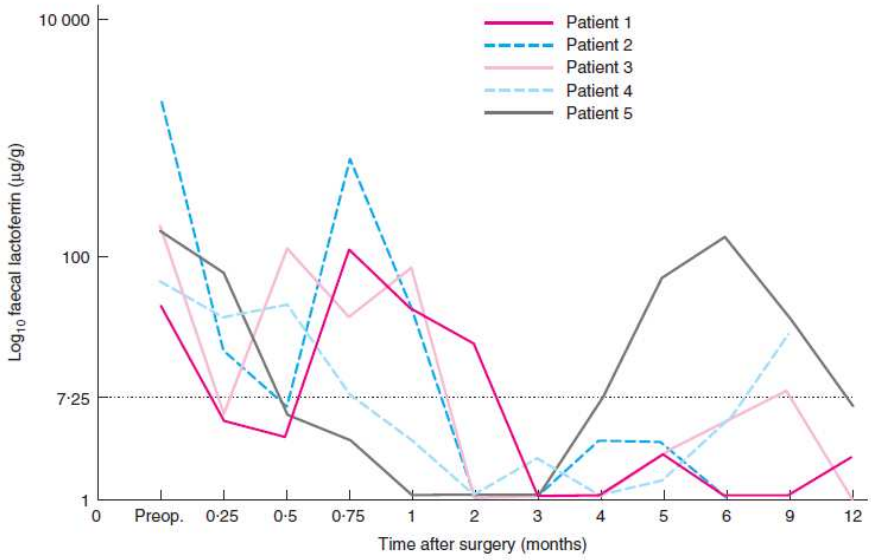

Fig. 3 Faecal lactoferrin concentration before and after surgery for five patients with a complicated postoperative recovery. The dotted line represents upper limit of normal

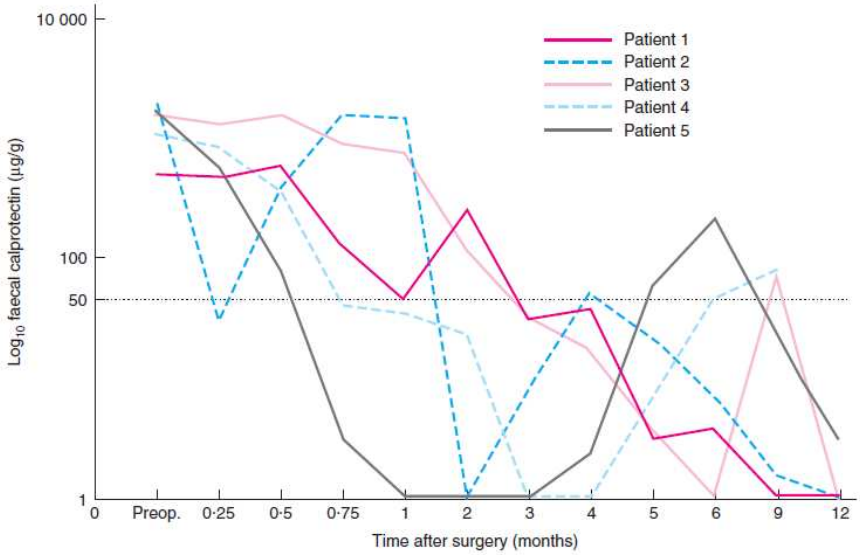

Fig. 4 Faecal calprotectin concentration before and after surgery for five patients with a complicated postoperative recovery. The dotted line represents upper limit of normal

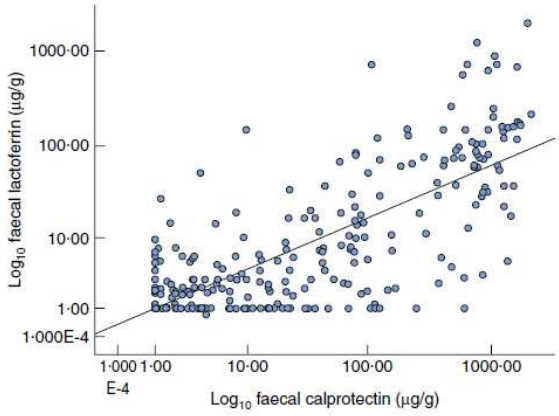

Fig. 5 Correlation between faecal calprotectin and lactoferrin in each collected stool specimen (Spearman's $r = 0{\cdot}71$, $P < 0{\cdot}001$)

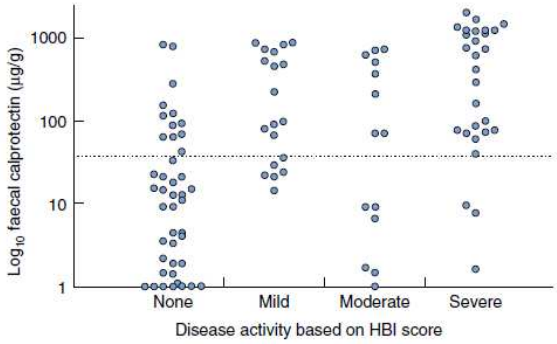

Fig. 6 Logarithmic distribution of faecal calprotectin related to the Harvey Bradshaw Index (HBI). The dotted line represents upper limit of normal

that FC and FL correlated significantly with each other ($r = 0{\cdot}71$; $P < 0{\cdot}001$) (*Fig. 5*)

Both FC and FL correlated significantly with the HBI definition of clinical activity ($r = 0{\cdot}53$, $P < 0{\cdot}001$ and $r = 0{\cdot}69$, $P < 0{\cdot}001$ respectively) (*Table 3*). The CRP level also showed a significant but weak correlation with clinical activity assessed by HBI ($r = 0{\cdot}33$, $P < 0{\cdot}001$). Based on the HBI score, 28 patients had severely clinically active disease (6 or more), 43 had inactive disease (3 or less), 19 had mildly active disease (4) and 14 had moderately active disease (5). Patients with severely clinically active disease had high FC, FL and CRP levels with a mean(s.e.) of 661·1(119·1) μg/g, 116·6(32·2) μg/g and 44·5(14·3) mg/l respectively, compared with 70·2(27·1) μg/g, 5·9(2·4) μg/g and 7·7(1·7) mg/l in the 43 patients with clinically inactive disease (*Table 3*). The distribution of FC and FL related to HBI is shown in *Figs 6* and *7*.

The 104 patients included 41 current smokers, 32 former smokers and 31 who had never smoked.

Table 3 Correlation between faecal and serum parameters and Harvey Bradshaw Index clinical activity

Clinical activity (Harvey Bradshaw Index score)	Faecal calprotectin levels (μg/g)	Faecal lactoferrin levels (μg/g)	C-reactive protein (mg/l)	Platelet count ($\times 10^9$/l)
None (≤ 3)	70·2(27·1)	5·9(2·4)	7·7(1·7)	311·7(13·7)
Mild (4)	333·7(78·9)	51·3(14·8)	14·6(5·2)	271·5(15·1)
Moderate (5)	242·4(79·2)	77·8(50·4)	9·7(2·5)	310·3(16·1)
Severe (≥ 6)	661·1(119·1)	116·6(32·2)	44·5(14·3)	311·0(17·8)

Values are mean(s.e.).

Table 4 Correlation between Harvey Bradshaw Index clinical disease activity and high and low levels of gut inflammation defined by faecal marker concentration

	No. of patients	Mean FC (μg/g)	Mean FL (μg/g)	HBI score ≤ 3 ($n = 43$)	HBI score 4–5 ($n = 33$)	HBI score ≥ 6 ($n = 28$)
FC < 100 μg/g (2 × ULN)	64	28		37	17	10
FC > 100 μg/g (2 × ULN)	40	737		6	16	18
FL < 14·5 μg/g (2 × ULN)	62		4	41	15	6
FL > 14·5 μg/g (2 × ULN)	42		127	2	18	22

FC, faecal calprotectin; FL, faecal lactoferrin; HBI, Harvey Bradshaw Index; ULN, upper limit of normal.

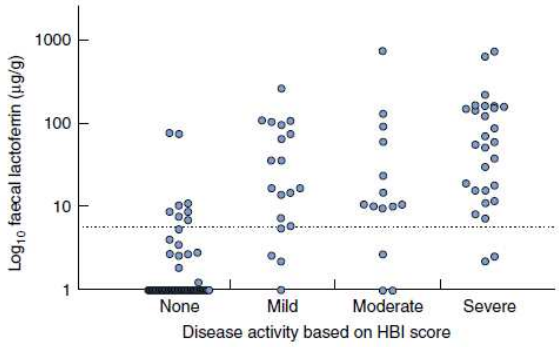

Fig. 7 Logarithmic distribution of faecal lactoferrin related to the Harvey Bradshaw Index (HBI). The dotted line represents upper limit of normal

Table 5 Faecal marker and serum marker concentrations in patients with and without endoscopic evidence of disease recurrence

	No endoscopic disease recurrence ($n = 18$)	Endoscopic disease recurrence ($n = 25$)
Harvey Bradshaw Index		
≤ 3	9	8
4–5	7	8
≥ 6	2	9
Faecal calprotectin (μg/g)*	295·0(82·7)	395·0(135·3)
Faecal lactoferrin (μg/g)*	76·8(36·8)	88·9(41·8)
Platelet count ($\times 10^9$/l)*	325·9(17·2)	331·8(14·4)
C-reactive protein (mg/l)*	16·0(5·9)	22·8(11·9)

*Values are mean(s.e.). $P = 0·082$ (Kruskal–Wallis test).

Using the Mann–Whitney U test, no significant difference was found between smokers and non-smokers in the faecal markers (FC, $P = 0·284$; FL, $P = 0·612$).

Twenty-three patients received corticosteroid therapy and 39 received azathioprine. There were no significant differences in FC or FL values in either group between those who received and those who did not receive the treatments.

The HBI and mean faecal marker levels were compared in high *versus* low levels of gut inflammation, defined by FC or FL values of more or less than twice the upper limit of normal (ULN) (100·0 and 14·5 μg/g respectively) (*Table 4*). Those who were asymptomatic (HBI score of 3 or less) had low mean FC and FL values demonstrating low levels of gut inflammation. Patients with high levels of clinical disease activity (HBI score of 6 or more) tended to have higher levels of FC and FL corresponding with high levels of gut inflammation. Those with HBI scores of 4–5 (clinically mild to moderate symptoms) were far more heterogeneous, with almost equal numbers of patients having high or low levels of gut inflammation defined as above. FC and FL therefore allowed identification of two groups with similar clinical severity indices but with very different biomarker levels of gut inflammation.

Of the 104 patients included in the cross-sectional study, 43 had undergone endoscopic assessment up to 4 weeks before the patient interview. Of these, 25 had endoscopic evidence of disease recurrence. HBI symptom scores,

mean(s.e.) values of FC, FL, platelet counts and CRP values for patients with and without endoscopic evidence of disease recurrence are shown in *Table 5*. There was no significant difference between the FC and FL values in those with endoscopic evidence of disease recurrence and those without ($P = 0{\cdot}676$ and $P = 0{\cdot}730$ respectively).

Discussion

This study has shown that quantitative measurement of FC and FL offers a valuable, non-invasive method of assessing symptomatic postoperative patients with Crohn's disease. Both tests have been demonstrated as reproducible and correlated well with one another. After uncomplicated ileocaecal resection for Crohn's disease, both markers normalized by 2 months. After this time, both remained within the normal range unless there was a recurrence of gut inflammation.

A previous report demonstrated that normalization of FC was associated not just with clinical remission but with mucosal healing[26]. This suggests that, in symptomatic postoperative patients, a single FC or FL measurement at 2 months or more after resection could identify genuine disease recurrence and help to target immunosuppressant therapy.

This study has not demonstrated that one faecal marker is superior to the other, and either can be reliably used as a single measurement to quantify intestinal inflammation in postoperative Crohn's disease. Those with low levels of FC or FL after resection who have symptoms are unlikely to have mucosal inflammation, so symptoms may be due to altered anatomy, bile salt malabsorption or functional bowel disorder. In these patients, conservative management with antimotility medications such as loperamide and cholestyramine should be considered. Symptomatic patients with high levels of FC or FL and hence a high degree of mucosal inflammation may benefit from agents such as thiopurines or anti-TNF-α antibodies.

Surprisingly, no significant difference was apparent in FC and FL concentrations between patients with endoscopic evidence of inflammation and those with normal endoscopic appearance. This conflicts with findings by Sipponen and colleagues[38]. Interestingly, D'Incà and co-workers[39] reported a significant correlation between endoscopic inflammation and FC, and between histological inflammation and FL, but not *vice versa*. However, it should be noted that they used a quantitative assay for FC but only a qualitative assay for FL. Previous research in patients with ulcerative colitis has suggested that FC correlates with degree of inflammation rather than disease extent[40], and that FC levels are significantly raised in active small bowel Crohn's disease[41]. Therefore it may be that patients with high FC or FL concentrations but normal endoscopy have active small bowel disease beyond the reach of the colonoscope. It is also worth noting that the time between colonoscopy and interview in the present study was up to 4 weeks. The longitudinal data demonstrated that there can be significant variation in FC and FL levels within this time; therefore, when comparing FC and FL concentrations with endoscopic appearance, the interval between the two is a limitation.

This study has also demonstrated that both FC and FL correlate more strongly than CRP or platelet count with disease symptom scoring according to the HBI, and may therefore give a more accurate reflection of intestinal inflammation than these commonly used laboratory markers.

In patients who had a complicated postoperative recovery, their FC and FL normalized again after they responded to therapy. Buderus and colleagues[36] have demonstrated that in a paediatric population FL could be useful in monitoring response to infliximab therapy when compared with the paediatric CDAI. Kolho and co-workers[42] have shown that, although FC decreases in line with clinical response in patients with active IBD treated with corticosteroids, it does not always fully normalize. This is likely to reflect the fact that corticosteroids do not induce full resolution of mucosal inflammation[43,44] that could be achieved by other therapeutic means such as azathioprine or infliximab[9,10,45–47].

The most novel finding of this study was the association between FC and FL levels and long-term active or inactive disease in postoperative patients. The cross-sectional study showed a high degree of correlation between clinical disease activity and high and low levels of gut inflammation defined by an FL or FC more or less than twice the ULN. In patients with no clinical disease activity (HBI score of less than 3), most patients had low levels of FC and FL (less than twice the ULN), and therefore low levels of gut inflammation. Those with very active disease (HBI score of 6 or more) generally had high levels of gut inflammation (FC and FL both over twice the ULN). However, those with mild to moderate symptoms of disease activity (HBI score of 4–5) formed a far more diverse group. This is important to recognize, as often these are the most challenging patients in which to distinguish inactive from active disease. The finding of an almost equal split between patients with high and those with low levels of gut inflammation within a similar spectrum of clinical disease activity suggests that a single measurement of FC and FL could allow treatment targeting between

immunosuppression for those with high concentrations of faecal granulocyte degradation products and symptomatic treatment for those with low concentrations.

This evidence favouring the routine use of faecal granulocyte markers in postoperative Crohn's is supported by the work of Orlando and colleagues[48]. They investigated the role of a single FC measurement in predicting disease recurrence in patients with asymptomatic Crohn's disease within a year of resection, finding an FC of 200 μg/l gave a sensitivity of 63 per cent and a specificity of 75 per cent. They therefore recommended that this value could be used as a tool to decide which patients should have colonoscopy after surgery.

Scarpa and co-workers[37] also carried out a cross-sectional study in a small cohort to assess FC and FL levels randomly at a median of 40·5 months after ileocolonic resection. Using the CDAI, they too demonstrated that some patients have high FC and FL levels after surgery even in clinical remission. However, because their cohort was smaller than the present study, they could not stratify clinical disease activity, nor compare FC and FL measurements. Furthermore, they did not have a cohort of patients with serial measurements of FC or FL from the time of surgery.

The limitation of all studies in this group of postoperative patients is the lack of a 'gold standard' test of disease recurrence. It is therefore hard to validate the use of any test fully. There is also a degree of subjectivity and bias associated with the use of clinical indices such as the CDAI and HBI, although clinical indices do reflect individual well-being, which in clinical practice is used to guide management.

Further trials are desirable to investigate targeted azathioprine or anti-TNF therapy in those with high FC or FL levels, and withdrawing or reducing immunosuppressant therapy in those with low FC or FL levels.

This study has demonstrated that non-invasive measurement of FC and FL may be useful in postoperative Crohn's disease, to assess whether the symptomatic patient has a recurrence of intestinal inflammation that may benefit from immunosuppressant therapy. The tests are inexpensive, reliable and are more accurate at predicting clinical disease activity than serum markers of inflammation or endoscopic appearance. FC and FL measurement can be recommended for routine use in postoperative patients to help target immunosuppressant therapy.

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References

1 Bernell O, Lapidus A, Hellers G. Risk factors for surgery and recurrence in 907 patients with primary ileocaecal Crohn's disease. *Br J Surg* 2000; **87**: 1697–1701.
2 Rutgeerts P, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology* 1990; **99**: 956–963.
3 Tytgat GN, Mulder CJ, Brummelkamp WH. Endoscopic lesions in Crohn's disease early after ileocecal resection. *Endoscopy* 1988; **20**: 260–262.
4 Wolff BG. Factors determining recurrence following surgery for Crohn's disease. *World J Surg* 1998; **22**: 364–369.
5 Borley NR, Mortensen NJ, Jewell DP. Preventing postoperative recurrence of Crohn's disease. *Br J Surg* 1997; **84**: 1493–1502.
6 Moskovitz D, McLeod RS, Greenberg GR, Cohen Z. Operative and environmental risk factors for recurrence of Crohn's disease. *Int J Colorectal Dis* 1999; **14**: 224–226.
7 Cottone M, Rosselli M, Orlando A, Oliva L, Puleo A, Cappello M *et al.* Smoking habits and recurrence in Crohn's disease. *Gastroenterology* 1994; **106**: 643–648.
8 Holzheimer RG, Molloy RG, Wittmann DH. Postoperative complications predict recurrence of Crohn's disease. *Eur J Surg* 1995; **161**: 129–135.
9 D'Haens G, Geboes K, Ponette E, Penninckx F, Rutgeerts P. Healing of severe recurrent ileitis with azathioprine therapy in patients with Crohn's disease. *Gastroenterology* 1997; **112**: 1475–1481.
10 D'Haens G, Geboes K, Rutgeerts P. Endoscopic and histologic healing of Crohn's (ileo-) colitis with azathioprine. *Gastrointest Endosc* 1999; **50**: 667–671.
11 Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 1976; **70**: 439–444.
12 Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980; **1**: 514.
13 Sostegni R, Daperno M, Scaglione N, Lavagna A, Rocca R, Pera A. Review article: Crohn's disease: monitoring disease activity. *Alimentary Pharmacol Ther* 2003; **17**(Suppl 2): 11–17.
14 de Dombal FT, Softley A. IOIBD report no 1: Observer variation in calculating indices of severity and activity in Crohn's disease. International Organisation for the Study of Inflammatory Bowel Disease. *Gut* 1987; **28**: 474–481.
15 Boirivant M, Leoni M, Tariciotti D, Fais S, Squarcia O, Pallone F. The clinical significance of serum C reactive

protein levels in Crohn's disease. Results of a prospective longitudinal study. *J Clin Gastroenterol* 1988; **10**: 401–405.
16 Cellier C, Sahmoud T, Froguel E, Adenis A, Belaiche J, Bretagne JF *et al*. Correlations between clinical activity, endoscopic severity, and biological parameters in colonic or ileocolonic Crohn's disease. A prospective multicentre study of 121 cases. The Groupe d'Etudes Therapeutiques des Affections Inflammatoires Digestives. *Gut* 1994; **35**: 231–235.
17 Saverymuttu SH, Peters AM, Lavender JP, Hodgson HJ, Chadwick VS. 111Indium autologous leucocytes in inflammatory bowel disease. *Gut* 1983; **24**: 293–299.
18 Saverymuttu SH, Peters AM, Crofton ME, Rees H, Lavender JP, Hodgson HJ *et al*. 111Indium autologous granulocytes in the detection of inflammatory bowel disease. *Gut* 1985; **26**: 955–960.
19 Saverymuttu SH, Peters AM, Lavender JP, Pepys MB, Hodgson HJ, Chadwick VS. Quantitative fecal indium 111-labeled leukocyte excretion in the assessment of disease in Crohn's disease. *Gastroenterology* 1983; **85**: 1333–1339.
20 Saverymuttu SH, Peters AM, Hodgson HJ, Chadwick VS. Assessment of disease activity in ulcerative colitis using indium-111-labelled leukocyte faecal excretion. *Scand J Gastroenterol* 1983; **18**: 907–912.
21 Tibble J, Teahon K, Thjodleifsson B, Roseth A, Sigthorsson G, Bridger S *et al*. A simple method for assessing intestinal inflammation in Crohn's disease. *Gut* 2000; **47**: 506–513.
22 Tibble JA, Sigthorsson G, Bridger S, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* 2000; **119**: 15–22.
23 Tibble JA, Sigthorsson G, Foster R, Forgacs I, Bjarnason I. Use of surrogate markers of inflammation and Rome criteria to distinguish organic from nonorganic intestinal disease. *Gastroenterology* 2002; **123**: 450–460.
24 Costa F, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C *et al*. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 2005; **54**: 364–368.
25 Bunn SK, Bisset WM, Main MJ, Golden BE. Fecal calprotectin as a measure of disease activity in childhood inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2001; **32**: 171–177.
26 Røseth AG, Aadland E, Grzyb K. Normalization of faecal calprotectin: a predictor of mucosal healing in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2004; **39**: 1017–1020.
27 Uchida K, Matsuse R, Tomita S, Sugi K, Saitoh O, Ohshiba S. Immunochemical detection of human lactoferrin in feces as a new marker for inflammatory gastrointestinal disorders and colon cancer. *Clin Biochem* 1994; **27**: 259–264.
28 Baveye S, Elass E, Mazurier J, Spik G, Legrand D. Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin Chem Lab Med* 1999; **37**: 281–286.
29 Guerrant RL, Araujo V, Soares E, Kotloff K, Lima AA, Cooper WH *et al*. Measurement of fecal lactoferrin as a marker of fecal leukocytes. *J Clin Microbiol* 1992; **30**: 1238–1242.
30 Kane SV, Sandborn WJ, Rufo PA, Zholudev A, Boone J, Lyerly D *et al*. Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. *Am J Gastroenterol* 2003; **98**: 1309–1314.
31 Sugi K, Saitoh O, Hirata I, Katsu K. Fecal lactoferrin as a marker for disease activity in inflammatory bowel disease: comparison with other neutrophil-derived proteins. *Am J Gastroenterol* 1996; **91**: 927–934.
32 Walker TR, Land ML, Kartashov A, Saslowsky TM, Lyerly DM, Boone JH *et al*. Fecal lactoferrin is a sensitive and specific marker of disease activity in children and young adults with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2007; **44**: 414–422.
33 Gaya DR, Lyon TD, Duncan A, Neilly JB, Han S, Howell J *et al*. Faecal calprotectin in the assessment of Crohn's disease activity. *QJM* 2005; **98**: 435–441.
34 Costa F, Mumolo MG, Bellini M, Romano MR, Ceccarelli L, Arpe P *et al*. Role of faecal calprotectin as non-invasive marker of intestinal inflammation. *Dig Liver Dis* 2003; **35**: 642–647.
35 Wassell J, Dolwani S, Metzner M, Losty H, Hawthorne A. Faecal calprotectin: a new marker for Crohn's disease? *Ann Clin Biochem* 2004; **41**: 230–232.
36 Buderus S, Boone J, Lyerly D, Lentze MJ. Fecal lactoferrin: a new parameter to monitor infliximab therapy. *Dig Dis Sci* 2004; **49**: 1036–1039.
37 Scarpa M, D'Incà R, Basso D, Ruffolo C, Polese L, Bertin E *et al*. Fecal lactoferrin and calprotectin after ileocolonic resection for Crohn's disease. *Dis Colon Rectum* 2007; **50**: 861–869.
38 Sipponen T, Savilahti E, Kolho KL, Nuutinen H, Turunen U, Färkkilä M. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. *Inflamm Bowel Dis* 2008; **14**: 40–46.
39 D'Incà R, Dal Pont E, Di Leo V, Ferronato A, Fries W, Vettorato MG *et al*. Calprotectin and lactoferrin in the assessment of intestinal inflammation and organic disease. *Int J Colorectal Dis* 2007; **22**: 429–437.
40 Røseth AG, Aadland E, Jahnsen J, Raknerud N. Assessment of disease activity in ulcerative colitis by faecal calprotectin, a novel granulocyte marker protein. *Digestion* 1997; **58**: 176–180.
41 Dolwani S, Metzner M, Wassell JJ, Yong A, Hawthorne AB. Diagnostic accuracy of faecal calprotectin estimation in prediction of abnormal small bowel radiology. *Aliment Pharmacol Ther* 2004; **20**: 615–621.
42 Kolho KL, Raivio T, Lindahl H, Savilahti E. Fecal calprotectin remains high during glucocorticoid therapy in children with inflammatory bowel disease. *Scand J Gastroenterol* 2006; **41**: 720–725.

43 Modigliani R, Mary JY, Simon JF, Cortot A, Soule JC, Gendre JP *et al*. Clinical, biological, and endoscopic picture of attacks of Crohn's disease. Evolution on prednisolone. Groupe d'Etude Thérapeutique des Affections Inflammatoires Digestives. *Gastroenterology* 1990; **98**: 811–818.
44 Olaison G, Sjödahl R, Tagesson C. Glucocorticoid treatment in ileal Crohn's disease: relief of symptoms but not of endoscopically viewed inflammation. *Gut* 1990; **31**: 325–328.
45 Lemann M, Mary JY, Colombel JF, Duclos B, Soule JC, Lerebours E *et al*. A randomized, double-blind, controlled withdrawal trial in Crohn's disease patients in long-term remission on azathioprine. *Gastroenterology* 2005; **128**: 1812–1818.
46 D'Haens G, Van Deventer S, Van Hogezand R, Chalmers D, Kothe C, Baert F *et al*. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: a European multicenter trial. *Gastroenterology* 1999; **116**: 1029–1034.
47 Rutgeerts P, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF *et al*. Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. *Gastroenterology* 2004; **126**: 402–413.
48 Orlando A, Modesto I, Castiglione F, Scala L, Scimeca D, Rispo A *et al*. The role of calprotectin in predicting endoscopic postsurgical recurrence in asymptomatic Crohn's disease: a comparison with ultrasound. *Eur Rev Med Pharmacol Sci* 2006; **10**: 17–22.

MINERVA

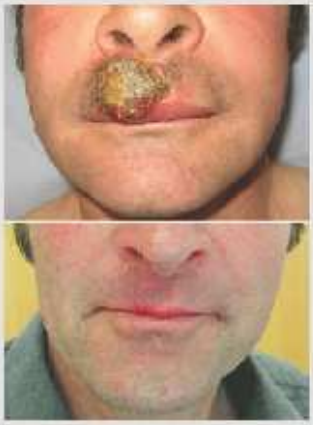
A 44 year old man with a two day history of fever, skin rash, and a rapidly progressive shortness of breath. Try the picture quiz in ENDGAMES, p 1337

A 48 week study of patients with mild cognitive impairment treated with donepezil, an acetylcholinesterase inhibitor, showed no significant improvements in the primary measures of cognition or of global function. And nearly all the measures of global impairment, cognition, and function remained unchanged. The authors suggest that the measures used may be insensitive to change in mild cognitive impairment. Patients, however, reported perceived benefits with donepezil (*Neurology* 2009;72:1555-61, doi:10.1212/01.wnl.0000344650.95823.03).

High energy war zone trauma, especially in blast injured amputees, lends itself to heterotopic ossification. A retrospective study of US troops from 2003 to 2006 identified a higher prevalence of heterotopic ossification in war wounded patients than in civilians who had sustained trauma. Risk factors in the military include age under 30, the presence of severe brain injury, and multiple injuries to the extremities, which points to systemic causes rather than a single origin (*Journal of Bone and Joint Surgery Am* 2009;91:1084-91, doi:10.2106/jbjs.h.00792).

Spanish intensive care specialists designed a pilot study to test their theory that subjecting patients to 100 ml of additional "dead space" after a two hour weaning trial with successful spontaneous breathing could predict readiness for extubation. The dead space was added to the endotracheal tube for 30 minutes. Patients who tolerated the test were extubated; those who didn't were given another six hours of ventilation. The test gave a sensitivity of 40.9%, a specificity of 97.7%, and a probability of extubation failure of 75.1% for patients who did not tolerate the test versus 9.3% for those tolerating it (*Critical Care* 2009;13:R56, doi:10.1186/cc7783).

Surgeons have highlighted a non-invasive method for identifying Crohn's disease recurrence in symptomatic patients, after ileocaecal resection. Thirteen patients were followed up after surgery for ~12 months with regular measurements of faecal calprotectin and lactoferrin. A second cohort of 104 postoperative patients provided a single stool sample. Those with severely clinically active disease had high levels of both markers, whereas those with clinically inactive disease had low levels. For those in between, recurrent inflammatory disease could be predicted by the level of faecal markers, and the markers were more accurate at predicting disease activity than were C-reactive protein, platelet count, or endoscopic appearance (*British Journal of Surgery* 2009;96:663-74, doi:10.1002/bjs.6593).



A 56 year old sheep farmer presented three weeks after being butted on the mouth by a lamb. A rapidly growing asymptomatic crusted nodule developed on the upper lip shortly afterwards. Orf was initially considered but no virus particles were detected on electron microscopy. Skin biopsy confirmed the clinical suspicion of a pyogenic granuloma, a benign vascular entity often related to minor trauma. Treatment with topical super-potent corticosteroid ointment (clobetasol propionate) led to complete resolution over subsequent weeks. The substantial vasoconstrictor effect of this medicine was used effectively, obviating the need for surgery at a cosmetically sensitive site.

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Patient consent obtained.

Cite this as: *BMJ* 2009;338:b2106

Many types of solid cancers have a lower incidence in people with Down's syndrome than in the general population, and this protection appears to be caused by an extra copy of one of the genes on chromosome 21 known as *Dscr1*. In a mouse model, this extra copy is sufficient to slow down cancer growth, and it seems to work in conjunction with another gene on chromosome 21 by interfering with the calcineurin signalling pathway that enables tumours to grow their own blood supply. Identification of this mechanism opens up a potential target for therapeutic intervention in cancer (*Nature* 2009; published online 20 May, doi:10.1038/nature08062).

A Hungarian psychiatrist called Laszlo Meduna was the first person to induce epileptic fits to try to influence the course of mental illness, in 1934 (*British Journal of Psychiatry* 2009;194:387-8, doi:10.1192/bjp.bp.108.062547). His intervention led to the introduction of electroconvulsive therapy. Initially he used injections of camphor, and then a cardiac stimulant (cardiazol) instead, which he found induced fits more quickly and more reliably. It was also cheaper. Meduna eventually concluded that cardiazol didn't cure schizophrenia, but it accelerated remission in cases with good prognosis. In keeping with the times, Meduna had neither a study design nor patient consent.

Serum gamma-glutamyltransferase is associated with incident cardiovascular disease and is a potential risk factor for disease mortality. But how does it relate to chronic heart failure? A study of almost 1000 patients found that the prevalence of raised gamma-glutamyltransferase is high in patients with chronic heart failure, and the level is associated with disease severity. Increased gamma-glutamyltransferase seems to be an independent predictor of death or need for heart transplantation, and as such can provide additional prognostic information especially in patients with mild heart failure (*Circulation Heart Failure* 2009; published online May 14, doi:10.1161/circheartfailure.108.826735).

Active community screening for hearing loss and delivery of hearing aids in a region of India has proved more expensive than passive screening and fitting of aids at a specialist hospital audiology department (US$152 v $122 per patient). The cost per disability-adjusted life year averted was around $900 in the community and $720 at the specialist unit level. One advantage of active screening in the community, however, is that it can be combined with the screening and management of other diseases, which doesn't happen in specialist units (*BMC Public Health* 2009;9:135, doi:10.1186/1471-2458-9-135).

Cite this as: *BMJ* 2009;338:b2112

Can Fecal Calprotectin or Lactoferrin Identify Postoperative Recurrence in Crohn's Disease?

Lamb CA, Mohiuddin MK, Gicquel J, et al. Fecal calprotectin or lactoferrin can identify postoperative recurrence in Crohn's disease. Br J Surg. 2009;96:663–674.

Fecal calprotectin and lactoferrin are sensitive markers of intestinal inflammation in IBD. They have been shown to correlate with clinical and endoscopic activity[1–3] and their prognostic value in detecting upcoming flares is increasingly being recognized.[4]

Lamb et al investigated fecal calprotectin and lactoferrin in two Crohn's disease (CD) patient cohorts having undergone ileocecal resection. In the first cohort 13 patients were prospectively followed for 1 year with repetitive fecal sampling (in total, 155 samples), whereas in the second cohort 104 patients (median 24 months after resection) were retrospectively analyzed based on a single stool sample. Fecal measurements were compared with the Harvey–Bradshaw Index (HBI), ileocolonoscopic findings, C-reactive protein (CRP), and platelet counts.

The authors found a normalization of the fecal markers within 2 months postoperatively in cases with uncomplicated disease course. Calprotectin as well as lactoferrin correlated significantly with HBI. Both fecal markers were found elevated in a high proportion of CD patients with severe clinical activity, whereas low levels were measured in patients with clinically inactive disease. In patients with mild to moderately clinically active disease, fecal calprotectin and lactoferrin were able to identify individuals with and without recurrent inflammatory disease. The fecal markers were more accurate at predicting clinical disease activity than CRP, platelet count, or endoscopic appearance. The authors concluded that both fecal calprotectin and lactoferrin could help to identify disease recurrence in symptomatic postoperative patients.

COMMENT

The therapeutic goal after surgery is to avoid early recurrence and reoperation in further follow-up. An endoscopic workup 3–6 months after surgery is an internationally accepted standard which helps to decide whether the actual treatment of the patients is appropriate.[5] So far, noninvasive markers such as CRP, blood leukocytes, and clinical activity indices frequently fail to detect endoscopic recurrence which precedes clinical activity.[5] Therefore, an accurate, noninvasive, and inexpensive marker would be very helpful in the postoperative setting. Meanwhile, numerous studies have demonstrated that fecal calprotectin and lactoferrin are sensitive to detect intestinal inflammation. The current study by Lamb et al adds new insights in the situation of postoperative CD monitoring, demonstrating that fecal calprotectin and lactoferrin correlate closely with HBI. It would indeed be attractive for patients and for economic reasons if repetitive determination of lactoferrin or calprotectin could help to decrease the frequency of ileocolonoscopies being performed for postoperative activity monitoring. However, several questions remain. Will the repetitive determination of fecal markers alter therapeutic decisions and thereby finally lead to a reduction of disease-inherent complications and further surgical procedures? Large prospective randomized long-term intervention studies will be necessary to evaluate this question. Frequently, the inflamed area around the anastomosis is limited, but will lead to restenosis over time if not appropriately treated. It will be of interest whether the fecal markers have sufficient sensitivity to detect these lesions and whether they ultimately are able to replace endoscopy in this context.

The authors showed that fecal markers correlated with postoperative complications and early postoperative recurrence; however, the number of prospectively followed patients was small ($n = 13$). An important finding of this study, however, is that the fecal markers may be helpful in the patient group with mild to moderate symptoms of disease activity (HBI score of 4–5). In this heterogeneous group of patients the determination of fecal markers permitted differentiation between inactive and active disease, which may have therapeutic consequences. The authors investigated 43 of 104 patients (41%) by ileocolonoscopy up to 4 weeks before patient interview, of which 25 had endoscopic recurrence. Of interest, they found no significant difference in the mean calprotectin or lactoferrin in

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the endoscopically active versus the inactive group. These findings stand in contrast with several studies demonstrating that the amount of fecal markers is proportional to the endoscopically assessed mucosal damage.[2,3,6–8] The number of patients having undergone ileocolonoscopies is too small to draw final conclusions; furthermore, the authors state that endoscopically undetected small bowel disease cephalad to the anastomosis could confound the findings. The authors report a good correlation between fecal markers and HBI in the postoperative setting. This good correlation also stands in contrast with other studies demonstrating that fecal markers correlated closest with endoscopic findings and that the correlation with clinical activity (assessed by the Crohn's Disease Activity Index) was limited.[2,6]

In summary, the results of Lamb et al are encouraging for the use of fecal markers in the postoperative surveillance of CD patients and should stimulate larger randomized prospective trials evaluating if disease monitoring by fecal markers alters clinical outcomes (complications, reoperations, quality of life) in the long term.

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REFERENCES

1. Langhorst J, Elsenbruch S, Koelzer J, et al. Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP and clinical indices. *Am J Gastroenterol*. 2008;103:162–169.
2. Schoepfer AM, Beglinger C, Straumann A, et al. Ulcerative colitis: correlation of the Rachmilewitz endoscopic activity index with fecal calprotectin, clinical activity, c-reactive protein, and blood leukocytes. *Inflamm Bowel Dis*. 2009;15:1851–1858.
3. Schoepfer AM, Beglinger C, Straumann A, et al. Fecal calprotectin correlates more closely with the simple endoscopic score for Crohn's disease (SES-CD) than CRP, blood leukocytes, and the CDAI. *Am J Gastroenterol*. 2009 [Epub ahead of print].
4. Gisbert JP, Bermejo F, Pérez-Calle JL, et al. Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse. *Inflamm Bowel Dis*. 2009;15:1190–1198.
5. Rutgeerts P, Geboes K, Vantrappen G, et al. Predictability of the postoperative course of Crohn's disease. *Gastroenterology*. 1990;99:956–963.
6. Sipponen T, Savilahti E, Kolho KL, et al. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. *Inflamm Bowel Dis*. 2008;14:40–46.
7. Schoepfer AM, Trummler M, Seeholzer P, et al. Discriminating IBD from IBS: comparison of the test performance of fecal markers, blood leukocytes, CRP, and IBD antibodies. *Inflamm Bowel Dis*. 2008;14:32–39.
8. Sipponen T, Kärkkäinen P, Savilahti E, et al. Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings. *Aliment Pharmacol Ther*. 2008;28:1221–1229.

Appendix

Appendix:

1) Patient information sheet: Cross-sectional study:

The Newcastle upon Tyne Hospitals NHS
NHS Trust

GASTROENTEROLOGY UNIT
Ward 48 Office
DR J C MANSFIELD
Tel/Fax: 0191 2820135
E.mail: john.mansfield@nuth.northy.nhs.uk

The Freeman Hospital
High Heaton
Newcastle upon Tyne
NE7 7DN

Tel: 0191 233 6161
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Patient Information Sheet

Project Title: A Cross Sectional Study of Inflammatory Markers in Stool Samples from Patients with Crohn's Disease

We would be grateful if you would spend a few minutes considering taking part in research being carried out at the Royal Victoria Infirmary. Please take time to read this sheet carefully.

Background

The assessment of Crohn's disease activity is never easy and following surgery for Crohn's disease it is particularly difficult. Crohn's disease may recur after operation so it is important to try to monitor the situation.

In this study we want to measure the markers of inflammation that can be detected in a small stool sample. In other situations this has been shown to reliably pick up the presence of bowel inflammation. The technique is available in some laboratories as a routine laboratory test.

How can you help?

Taking part in the research will involve giving one small stool sample (pea sized) after a routine clinic visit at which routine blood tests are taken. This test is stable so samples can be sent to us in a supplied secure container by post.

A small fraction of the stool sample will be kept frozen for potential analysis in the future, if new markers become available.

This research will not have any direct involvement in the routine treatment of your condition and we will keep any inconvenience down to a minimum. Your personal details and all the above information will be recorded and confidentially coded.

The results of your individual test will be returned to your clinic when it has been processed and your doctor can let you know what it showed at a future clinic visit. But it is unlikely to be useful in your management until this research project is completed.

The Newcastle upon Tyne Hospitals NHS
NHS Trust

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What next?

We hope you will decide to take part in the research and we would be grateful if you could sign the consent form included and send us both this and your stool sample in the enclosed pre-paid envelope.

If any questions do arise later please do not hesitate to get in touch with us. You may withdraw or not participate from the study at anytime without affecting your medical care.

Thank you for taking time to read this sheet.

Yours,

Dr John Mansfield
Consultant Gastroenterologist
Royal Victoria Infirmary
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Tel: 0191 2820135

Mr M Khalid Mohiuddin
Research Fellow Gastroenterology
Royal Victoria Infirmary
Newcastle upon Tyne
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2) Patient consent form: Cross-sectional study:

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NHS Trust

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CONSENT FORM

Project Title: A Cross Sectional Study of Inflammatory Markers in Stool Samples from Patients with Crohn's Disease

Name: DOB: Hospital Number:

Please initial box

1. I confirm that I have read and understand the information sheet dated 15.02.06 (version 2.2) for the above study and have had the opportunity to as questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from this research group where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to donate a stool sample to be used to investigate the disease activity. ☐
5. I understand a small fraction of the stool sample will be kept frozen for potential analysis in the future if new markers become available ☐

The Newcastle upon Tyne Hospitals NHS
NHS Trust

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Newcastle upon Tyne
NE7 7DN

Tel: 0191 233 6161
Fax: 0191 213 1968

6. I agree to take part in the above study. ☐

Please sign the two copies of this form- one for you to keep it and one to be returned to us.

Name of Patient	Date	Signature
Name of person taking consent	Date	Signature

If you have any further queries please feel free to contact:

Dr J Mansfield
Consultant Physician and Senior Lecturer
Royal Victoria Infirmary
Newcastle
Tele: 0191 2820135

Or

Mr M K Mohiuddin
Research Fellow
c/o Dr Mansfield's
Secretary

3) Patient information sheet: Longitudinal study:

The Newcastle upon Tyne Hospitals NHS
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Fax: 0191 201 0155

Patient Information Sheet

Project Title: A Study to Define The Post Operative Course of Inflammatory Markers in Stool Samples

We would be grateful if you would spend a few minutes considering taking part in research being carried out at The Royal Victoria Infirmary. Please take time to read this sheet carefully.

Background

The lining of the bowel heals quickly after bowel surgery. In some patients who have Crohn's disease there can be recurrent inflammation.

In order to be able to use stool samples to identify recurrent inflammation we want to define the usual post operative course of inflammatory markers in stool samples.

We aim to follow about 40 patients who have had recent bowel surgery (Crohn's disease, bowel cancer or appendix operation) for 12 months with a series of monthly stool samples.

This may show that the behaviour of the Crohn's disease can be identified at an early stage post operatively without unpleasant colonoscopies etc.

The result of your individual tests will be returned to your clinic when it has been processed so your doctor can let you know what it showed at a future clinic visit.

How you can help?

Taking part in the research will involve giving small stool samples (pea sized) after a routine clinic visit at which routine blood tests are taken. We will also ask you to post back a sample at monthly intervals over 12 months in secure containers and in pre paid packages. This test is stable so samples can be sent to us in a supplied secured container by post, after which they will be tested in the laboratory.

A small fraction of the stool sample will be kept frozen for potential analysis in the future, if new markers become available.

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This research will not have any direct involvement in the routine treatment of your condition and we will keep any inconvenience down to a minimum. Your personal details and all the above information will be recorded and confidentially coded.

What next?

We hope you will decide to take part in the research, we would be grateful if you could sign the consent form included and send us both this and your first stool sample in the enclosed prepaid envelope. We will send you your next sample kit in a month's time with a prepaid return container and envelope.

If any questions do arise later please do not hesitate to get in touch with us.

You may withdraw or not participate from the study at anytime without affecting your medical care.

Thank you for taking time to read this sheet.

Yours,

Dr John Mansfield
Consultant Gastroenterologist
Royal Victoria Infirmary
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Mr M Khalid Mohiuddin
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4) Patient consent from: Longitudinal study:

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CONSENT FORM

Project Title: A Study to Define The Post Operative Course of Inflammatory Markers in Stool Samples

Name: DOB: Hospital Number:

Please initial box

1. I confirm that I have read and understand the information sheet dated 15/02/06 (version 3.2) for the above study and have had the opportunity to as questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from this research group where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to donate a stool sample once a month for 12 months to be used to investigate the amount of inflammation in the bowel. ☐
5. I understand a small fraction of the stool sample will be kept frozen for potential analysis in the future if new markers become available ☐

The Newcastle upon Tyne Hospitals NHS
NHS Trust

Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne
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Tel: 0191 233 6161
Fax: 0191 201 0155

6. I agree to take part in the above study. ☐

Please sign the two copies of this form- one for you to keep it and one to be returned to us.

Name of Patient	Date	Signature
Name of person taking consent	Date	Signature

If you have any further queries please feel free to contact:

Dr J Mansfield
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Or

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Research Fellow
c/o Dr Mansfield's
Secretary