Neutrophil Activation in Inflammatory Bowel Disease

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Abstract

Inflammatory Bowel Disease (IBD) is an inflammatory condition in which neutrophils play an important role. Colonoscopy with biopsy is thought to be the best method for evaluating inflammation location, extent, and severity. However, the invasiveness of endoscopic examinations represents a strong limitation to their frequent use. Various studies have described faecal markers as powerful biomarkers of inflammation of the intestinal mucosa in patients with IBD. This thesis details the use of calprotectin and myeloperoxidase (MPO) as biomarkers of disease severity in patients with IBD, and aims to assess whether MPO is a superior biomarker to calprotectin. Abundant levels of both calprotectin and MPO are found in neutrophils.

A total of 500 patients were recruited into the evaluation of Novel Biomarkers in Inflammatory Bowel Disease project cohort. However, only 100 of the initial stool samples from patients with or without IBD were used for this research. The samples had been stored at -80°C for up to one year. A short extraction procedure using 100 mg of faeces and approximately 4.9 mL of the appropriate extraction buffer was carried out. Levels of calprotectin and MPO protein were then measured by sandwich enzyme-linked immunosorbent assay (ELISA). The peroxidase activity of MPO was also measured using 3,3',5,5'-tetramethylbenzidine (TMB) as a reducing substrate. TMB forms a blue product when it reacts with peroxidase enzymes such as MPO. The resulting colour change was read on a microplate reader at a wavelength of 630 nm.

Levels of calprotectin, MPO protein, and MPO TMB activity were significantly higher in IBD patients compared to controls. There were significant correlations between calprotectin, MPO protein and MPO TMB activity (p < 0.001). Levels of calprotectin and MPO protein correlated significantly with endoscopic disease severity in patients with CD (r = 0.487, p = 0.001, n = 41, r = 0.483, p = 0.001, n = 41, respectively) and UC (r = 0.677, p << 0.001, n = 35, r = 0.552, p < 0.001, n = 35, respectively). Consequently, both calprotectin and MPO protein were able to discriminate between IBD patients with inactive and high disease severity. However, MPO TMB activity failed to correlate with disease severity in CD and UC patients (r = 0.303, p = 0.054, n = 41; r = 0.258, p = 0.134, n = 35, respectively).

The results obtained from this research show that calprotectin and MPO protein correlate strongly with each other. There was also a strong correlation between MPO protein and disease severity, and MPO could successfully distinguish between inactive and high disease severity in CD and UC patients. This suggests that MPO may be useful in the diagnosis and follow-up of patients with IBD. Although, the relationships between MPO TMB activity and disease severity in patients with CD and UC were not significant results were still very promising. This assay could prove to be a faster and more cost effective approach to aid in the diagnosis and follow up of patients with IBD in the future.
However, further development and optimisation of the MPO ELISA and MPO TMB assay is required to validate the results from this research.
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<th>Definition</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
</tr>
<tr>
<td>CDEIS</td>
<td>Crohn’s Disease Endoscopic Index of Severity</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EPX</td>
<td>Eosinophil protein X</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
</tr>
<tr>
<td>FC</td>
<td>Faecal Calprotectin</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HBI</td>
<td>Harvey-Bradshaw Index</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>IC</td>
<td>Indeterminate Colitis</td>
</tr>
<tr>
<td>IUC</td>
<td>Idiopathic Ulcerative Colitis</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MgCl</td>
<td>Magnesium Chloride</td>
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<tr>
<td>mL</td>
<td>millilitre</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
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<tr>
<td>NaAc</td>
<td>Sodium Acetate</td>
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<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti Inflammatory Drug</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PGA</td>
<td>Physician’s Global Assessment</td>
</tr>
<tr>
<td>pM</td>
<td>picomolar</td>
</tr>
<tr>
<td>PNP</td>
<td>P-nitrophenyl phosphate disodium salt</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>SCCAI</td>
<td>Simple Clinical Colitis Activity Index</td>
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<tr>
<td>SCRP</td>
<td>Serum C-reactive protein</td>
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<tr>
<td>SES-CD</td>
<td>Simple Endoscopic Score for Crohn’s Disease</td>
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<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
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<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
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<tr>
<td>μM</td>
<td>micromolar</td>
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1 Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is the name of a group of disorders that cause the intestine to become inflamed (red and swollen). The inflammation can last for a long period of time and is usually recurring. Rates of IBD have increased exponentially in industrialised nations over the last 50 years with New Zealand having one of the highest incidence rates in the world (1). IBD is primarily a disease of young adults with onset typically between the ages of 15–35 years. Common symptoms of IBD include abdominal cramps and pain, bloody diarrhoea, severe urgency to pass a bowel motion, fever, loss of appetite, weight loss and anaemia (2).

Inflammatory bowel disease (IBD) consists of Ulcerative colitis (UC), Crohn’s disease (CD) and indeterminate colitis (IC) (3). UC is a condition in which the inflammatory response and morphologic changes remain confined to the colon. The rectum is involved in 95% of patients, with variable degrees of proximal extension. Inflammation is limited primarily to the mucosa and consists of continuous involvement of variable severity with ulceration, edema, and hemorrhage along the length of the colon. The characteristic histologic findings are acute and chronic inflammation of the mucosa by polymorphonuclear leukocytes and mononuclear cells, crypt abscesses, distortion of the mucosal glands, and goblet cell depletion. CD, in contrast to UC, can involve any part of the gastrointestinal tract from the oropharynx to the perianal area. Diseased segments frequently are separated by intervening normal bowel, leading to the term "skip areas." Inflammation can be transmural, often extending through to the serosa, resulting in sinus tracts or fistula formation. Histologic findings include small superficial ulcerations over a Peyer’s patch (aphthoid ulcer) and focal chronic inflammation extending to the submucosa, sometimes accompanied by noncaseating granuloma formation. The most common location is the ileocecal region, followed by the terminal ileum alone, diffuse small bowel, or isolated colonic disease in decreasing order of frequency (4). Figure 1.1 illustrates the human digestive system.
IBD is characterised by periods of remission with recurrent episodes of symptom exacerbation because of acute intestinal inflammation. The spectrum of IBD includes patients who remain in remission, those who have recurrent periods of disease followed by remission, and those who have continuous active disease (5). Currently the gold standard for assessing intestinal inflammation is endoscopy with biopsy sampling. Unfortunately, the invasiveness of endoscopic examinations represents a strong limitation to their frequent use, especially in paediatric patients (6). Furthermore, the site of inflammation is not always reached by endoscopy as in the case of small bowel disease in CD (7).

1.2 Pathogenesis of inflammatory bowel disease

UC and CD are precipitated by a complex interaction of environmental, genetic, and immunoregulatory factors (8). Higher rates of IBD are seen in northern, industrialized countries, with greater prevalence among Caucasians and Ashkenazic Jews. Racial gaps are closing, indicating that environmental factors may play a role. Regardless of the underlying genetic predisposition, a growing body of data implicates a dysfunctional mucosal immune response to commensal bacteria in the pathogenesis of IBD, especially CD (9). Possible triggers include a chronic inflammatory response precipitated by infection with a particular pathogen or virus or a defective mucosal barrier. The
characteristic inflammatory response begins with an infiltration of neutrophils and macrophages, which then release chemokines and cytokines. These in turn exacerbate the dysfunctional immune response and activate T helper cells in the gut mucosa, respectively associated with CD and, less conclusively, with UC (10).

The over-generation of reactive oxygen species (ROS) is also thought to play a large role in the pathogenesis of IBD (11). ROS are natural by-products of the normal metabolism of oxygen. To regulate the destructive effects of ROS, vital tissues are equipped with an intricate antioxidant defence system. Oxidative stress arises when there is a marked imbalance between the production of ROS and their removal by antioxidants. Previous studies demonstrated that peripheral blood monocytes and isolated intestinal macrophages from patients with IBD produce free radicals. In active IBD high numbers of peripheral neutrophils are present in the gut, which are capable of generating large amounts of superoxide. A growing body of evidence indicates that ROS, such as peroxide anion, hydrogen peroxide (H₂O₂) and hypochlorous acid, are not merely by-products of the inflammatory process, but they are actually involved in its pathogenesis (12).

1.3 Promising faecal markers in inflammatory bowel disease

Laboratory markers have been investigated in IBD for diagnostic and differential diagnostic purposes, for assessment of disease activity and risk of complications, for prediction of relapse, and for monitoring the effect of therapy. An ideal marker should have many qualities. It should be easy and rapid to perform, cheap, and reproducible between patients and laboratories. The ideal marker should furthermore be able to identify individuals at risk for the disease and should be disease specific; it should be able to detect disease activity and monitor the effect of treatment; and finally it should have a prognostic value towards relapse or recurrence of the disease (13). If the ideal marker exists for IBD, it would greatly facilitate the work of gastroenterologists and surgeons treating these patients. To date no single marker has proven to possess all the above listed qualities.

However, faecal markers seem promising and may be more specific in detecting gut inflammation in patients with IBD. The faecal stream is in direct contact with the intestinal mucosa, compared with the blood stream which is anatomically more distant. This should lead to a more sensitive measurement of inflammation for faecal tests compared with serum tests. Additionally, serum markers are more likely to be elevated in non-gastrointestinal inflammatory diseases than faecal markers (14). Neutrophil markers of inflammation reflect the presence and/or activation of leukocytes regardless of cause; therefore a positive result needs to be interpreted in its clinical context along with other diagnostic investigations. A number of neutrophil derived proteins present in stool have been studied, including faecal calprotectin, myeloperoxidase, lactoferrin, lysozyme and elastase (2-78).
1.4 Neutrophils

Neutrophils are the most abundant type of white blood cells in humans and form an essential part of the innate immune system. Neutrophils are normally found in the blood stream and are one of the first inflammatory cells to migrate towards the site of inflammation (15).

Our understanding of the role of the neutrophil has changed fundamentally over recent years. The initial perception of the neutrophil playing a passive role and merely responding to external signals has now been replaced by an appreciation that activated neutrophils can perform most (if not all) of the functions of numerous inflammatory diseases such as, IBD, inflammatory arthritis and chronic obstructive pulmonary disease. In some of these conditions neutrophils appear to have been inappropriately activated to release tissue damaging molecules (such as proteases) or alternatively, molecules that can promote inflammation such as chemoattractants, cytokines, or oxidants (17).

McCarthy et al. (18) hypothesised that activated neutrophils would circulate in the blood of IBD patients. The study showed that in patients with active IBD and in a small number of patients with other assorted untreated inflammatory diseases, that the number of polarized neutrophils is increased compared to normal controls. The morphological changes seen in neutrophils from IBD and inflammatory disease patients resembled those observed when normal bloods are incubated with chemotaxins in vitro. Polarization is just one of many responses which occur more or less simultaneously when neutrophils are activated, the simplest interpretation of these results is that the polarised neutrophils represent cells that have been activated in vivo, a finding which is consistent with the increased adherence of IBD neutrophils in vivo, and the enhanced release of leukotriene B4 (a leukotriene involved in inflammation) by peripheral blood neutrophils from IBD patients. The biomarkers selected to study in this research were calprotectin and myeloperoxidase and abundant levels of both of these proteins are found in neutrophils.

1.4.1 Myeloperoxidase

Myeloperoxidase (MPO) is an enzyme that functions in the oxygen-dependent killing of microorganisms, and is released from the primary granules of neutrophils during acute inflammation (3). The concentration of MPO is also proportional to the number of neutrophils within that region (19). MPO has long been considered a key constituent of the neutrophil cytotoxic armament by catalysing the formation of hypochlorous acid, a potent oxidant with bacterial activity in vitro (20). MPO produces hypochlorous acid from hydrogen peroxide and chloride anion during the neutrophils respiratory burst. It requires heme as a cofactor, and oxidises tyrosine to tyrosine radical using hydrogen peroxide as an oxidising agent. Hypochlorous acid and tyrosyl radical are cytotoxic, so they
can be used by the neutrophil to kill bacteria and other pathogens (21). MPO has been observed both in the intestinal mucosa (22-24), and in the gut lavage, and has been shown to correlate with disease activity (25), and have the potential to monitor treatment outcome (26).

### 1.4.2 Calprotectin

Numerous studies have assessed the role of calprotectin as a biomarker for IBD. Calprotectin is a calcium-binding protein that inhibits metalloproteinases, has antibacterial and antifungal activities and induces apoptosis in malignant and non-malignant cell cultures (27). Calprotectin constitutes 60% of neutrophil cytosolic proteins and is an abundant protein found in all body fluids in proportion to the degree of inflammation. Calprotectin has many clinical advantages; it is resistant to bacterial degradation in the gut and is stable in stool for up to one week at room temperature, allowing delays in transporting the sample to the laboratory. Furthermore, calprotectin can be readily quantified using enzyme-linked immunosorbent assay (ELISA) (3). Notably, random stool samples of <5 g show calprotectin concentrations equivalent to 24-hour homogenised specimens, demonstrating that calprotectin is uniformly scattered throughout the faeces (28).

### 1.5 Endoscopic and clinical disease activity scores

#### 1.5.1 Simple Endoscopic Score for Crohn’s Disease (SES-CD)

Several publications in paediatric and adult cohorts have demonstrated significant correlations between faecal markers and the severity of mucosal inflammation. Previous studies have demonstrated a good correlation between faecal calprotectin and the Crohn’s Disease Endoscopic Index of Severity (CDEIS). However, the CDEIS is cumbersome to complete and time-consuming in clinical practice; therefore the Simple Endoscopic Score for Crohn’s disease (SES-CD) was developed and validated in 2004. The SES-CD demonstrated a strong correlation with the CDEIS (r = 0.920) and an excellent inter-observer reliability (κ coefficients 0.791 - 1.000). Compared with the CDEIS the SES-CD is much easier to perform and thus more frequently used in clinical practice (29).

#### 1.5.2 Rachmilewitz score for Ulcerative colitis

The most frequently applied scores for evaluating endoscopic activity in UC are the Rachmilewitz Index and the Mayo Score. Both contain clinical and endoscopic items. The Rachmilewitz score has the advantage that the clinical as well as the endoscopic part can be used separately; furthermore, it is easy to calculate and increasingly used in clinical trials (30). Rachmilewitz and colleagues developed an activity index that included 7 variables; number of stools per day, blood in stools, Physician’s Global Assessment (PGA), abdominal pain/cramping, extraintestinal manifestations, erythrocyte sedimentation rate, and haemoglobin. Scores range from 0-29, with higher scores indicating more
severe disease activity. The scoring system was later validated in one trial that defined remission as four or fewer points. Investigators also utilised a scoring tool to evaluate endoscopic appearance of the mucosa (Rachmilewitz Endoscopic Index). The Endoscopic Index included four items: granulation scattering reflected light, vascular pattern, vulnerability of mucosa, and mucosal damage (mucus, fibrin, exudates, erosions, and ulcers) (31).

1.5.3 Crohn’s Disease Activity Index (CDAI)

Several studies have also demonstrated strong correlations between faecal markers and clinical activity indices. The Crohn’s Disease Activity Index (CDAI) was developed in the 1970s to assess the degree of illness in individuals with CD and has since been used widely in clinical trials of the condition. However, the need for a 7-day patient diary has been a major drawback of the CDAI. It is cumbersome and is occasionally filled in by a patient at one time rather than each day. In addition, the response to rapid clinical changes tends to be buffered by the 7-day collection period. The body weight variable is cumbersome, a minor influence on calculated CDAI values, and at times misleading. In recognition of these problems and to simplify computation, Harvey and Bradshaw modified the CDAI to create their index (the Harvey-Bradshaw Index [HBI]) by using only a single day’s reading for diary entries and excluding 3 variables body weight, hematocrit, and the use of lomotile or narcotics for diarrhoea, otherwise definitions of variables remain essentially the same. Harvey and Bradshaw presented a scattergram comparing HBI and CDAI in 112 patients. On the basis of a relatively high correlation coefficient, 0.93, they concluded, “Essentially the same information appears to be given by both methods” (32).

1.5.4 Simple Clinical Colitis Activity Index (SCCAI)

The Simple Clinical Colitis Activity Index (SCCAI) is used to assess disease severity in patients with UC, and is a validated symptom based index (score 0-19) which has good correlations with more complicated disease activity indices (33). The SCCAI was evaluated in a group of patients, with continuing assessment through the course of the disease, by comparison with a complex disease activity index derived from a combination of clinical and laboratory data, and with five laboratory markers of disease activity: haemoglobin, hematocrit, platelet count, erythrocyte sedimentation rate and serum albumin. Patients completed a questionnaire covering the clinical criteria of both indices and the scores were calculated when laboratory data became available. Comparison of indices and laboratory data was performed by Spearman rank correlation; the significance was $p < 0.05$ (34). For the purpose of this research the SES-CD and Rachmilewitz Endoscopic Index were used to assess endoscopic disease severity, and the HBI and SCCAI indices were used to assess clinical disease activity. These results will then be correlated with the levels of calprotectin and MPO.
1.6 Diagnosing inflammatory bowel disease

In routine clinical practice, gastroenterologists are often faced with the diagnostic difficulty of differentiating between irritable bowel syndrome (IBS) and those with intestinal pathology, particularly IBD. Many symptoms are common in both conditions, such as diarrhoea, abdominal pain and bloating, whereas rectal bleeding and systemic illness will increase the likelihood of IBD. This clinical differentiation remains problematic, and many patients are investigated extensively with invasive endoscopic imaging to make a diagnosis of exclusion. This has a significant impact for both health costs and exposing patients to the inherent risks associated with such procedures.

The use of non-invasive faecal markers in the diagnosis of IBD has shown to be promising in previous research. Many studies have dealt with the role of calprotectin in IBD diagnosis and follow-up. The leukocyte proteins calprotectin, lactoferrin, lysozyme, myeloperoxidase, and PMN-elastase were compared in faecal samples of three consecutive faeces in 40 healthy persons, 39 patients with chronic IBD and 40 patients with IBS (35). From this comparison, concentrations of all of the faecal leukocyte markers in IBS were found to be in the range of healthy patients. Moreover, faecal PMN-elastase and calprotectin differentiated between chronic IBD and IBS and correlated with the severity of inflammation.

A comparable study was conducted to evaluate faecal neutrophil-derived proteins in identifying intestinal inflammation (36). The sensitivity and specificity of calprotectin for IBD were 93% and 100%, respectively. In contrast, the respective diagnostic values for lactoferrin and polymorphonuclear neutrophil-elastase were 82% and 100% and 84% and 87%, respectively. Simultaneous determination of several biomarkers did not seem to improve the diagnostic power compared with each individual faecal neutrophil-derived protein. In conclusion, the results implicated an advantage of calprotectin over lactoferrin and PMN-elastase in the detection of intestinal inflammation provoked by IBD, when compared with the non-inflammatory condition, IBS. However, the validity of this data was flawed due to the limited sample size and selection bias which existed in this study cohort. Selection of patients with a strong suspicion of IBD, as also indicated by the exceptionally high number of patients suffering from IBD when compared with IBS in the overall patient cohort, could have positively influenced the diagnostic precision of the tests applied.

Similarly, another study was conducted to assess faecal excretion of calprotectin as an index of intestinal inflammation and its potential use as a screening test to discriminate between patients with CD and those with IBS (37). The results indicated that calprotectin concentrations were elevated in the majority of patients with established CD, and the method had a high sensitivity and specificity in prospectively discriminating between patients presenting with CD and IBS.
Tibble *et al.* (38) investigated 602 new referrals to a gastroenterology clinic with symptoms suggestive of IBS or organic disease such as IBD. A variety of different tests were performed on these patients including calprotectin, intestinal permeability studies, Rome I criteria, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP); the final diagnosis was made based only on radiology and/or endoscopy. Of the tests studied, a calprotectin concentration of >10 mg/L was most useful in the prediction of organic disease with a sensitivity of 89%, and specificity of 79%.

The diagnosis of paediatric IBD is difficult and delay to diagnosis is common. A recent study aimed to study faecal calprotectin (FC) in children with IBD and compare its diagnostic accuracy with blood parameters (39). The results suggest that the strength of FC as a measure of mucosal inflammation is that it can be detected at levels of disease activity insufficient to cause increases in systemic inflammatory markers. However, the authors concluded that FC is not a specific test for IBD, and concentrations can be increased in patients with other conditions such as gastrointestinal infection, coeliac disease, and non-steroidal anti-inflammatory drug (NSAID)-induced enteropathy (40). Infective causes should be excluded before calprotectin is measured in faeces. These results are comparable to those published previously (41, 42), in demonstrating blood abnormalities are common in most but not all patients at diagnosis. Another study found that measuring FC concentrations is a useful screening tool for identifying patients who are most likely to need endoscopy for suspected IBD. However, the discriminative power to safely exclude the disease was significantly better in studies of adults than in studies of children and teenagers (43).

Furthermore, numerous studies have compared calprotectin concentrations in patients with IBD versus controls and found significantly higher concentrations in IBD patients, particularly in those with active disease (7, 24, 35, 37, 44-50). Overall the evidence suggests that this marker may be useful in diagnosing IBD. However, there appeared to be limited literature assessing the use of the marker MPO as a diagnostic tool in IBD.

1.7 Disease severity

In order to determine the degree of inflammatory activity, it is important to monitor patients’ clinical evolution and adjust their therapy (51). Various indices are used to evaluate the activity of the disease, which differ from each other in terms of being more subjective (clinical), more objective (endoscopic-histological) or a combination of the two. However, despite the different indices available, there is little consensus in the literature as to which is the most valid. Several authors consider a colonoscopy with biopsy to be the best means of evaluating inflammation location, extent, and severity; however, this is an invasive method and carries risks of complications (6, 13, 43, 48, 50, 52, 53). Various studies have described faecal markers as powerful biomarkers in inflammation of the intestinal mucosa in patients with IBD (6, 7, 13, 19, 23-26, 28-78). There are several reasons why laboratory
Markers have been studied in IBD in the past decades: firstly, to gain an objective measurement of disease activity as symptoms are often subjective; and secondly, to avoid invasive (endoscopic) procedures which are often a burden to the patient.

In patients with active IBD, calprotectin values can vary between 200 mg/kg and 20,000 mg/kg (45, 46, 54, 55). Many authors have claimed that calprotectin concentrations correlate closely with more histological evaluation than with macroscopic findings, suggesting that this biological marker is more sensible than endoscopy in evaluating IBD activity (54). Recent studies have shown that there is a significant correlation between calprotectin concentrations and the severity of inflammation (49–54). Some studies indicated that calprotectin determination appears to reflect disease activity in UC more accurately than CD (55, 56). As an example, Costa et al. (40) found that FC concentrations above 50 µg/g more accurately correlated with the colitis activity index than the CDAI. However, this could have been due to the fact that CDAI is mostly a clinical score and is not sensitive enough to detect subclinical activity of the disease, which is known to occur rather frequently in CD. Nevertheless, several studies could not demonstrate a correlation between FC and clinical activity of CD (57, 58).

Xiang et al. (50) also found that FC concentrations were significantly higher in the patients with active UC than in patients with inactive UC and in the controls. In addition, the results showed that FC concentration was higher in the patients with inactive UC than in the controls.

Conversely, Gaya et al. (59) found that FC in patients with CD gives a reliable indication of the presence or absence of active inflammation, and an accurate reflection of the extent and/or severity of the inflammation. Tibble et al. (37) found there was a significant, albeit weak, correlation between FC and CDAI. However, it is important that these results are interpreted with caution due to the numerous problems that exist with the CDAI. Many variables on the score are subjective, identifying symptoms which are often suffered by patients with IBS, in addition to those with IBD. A large proportion of the score depends on the patients’ perception of the disease, and this can be influenced by the euphoric effect of corticosteroids used in the treatment of CD (60). Also, substantial inter-observer variability can occur when different observers review the same case notes to calculate the CDAI. Thus, it is not surprising that previous studies have found no correlation or only weak correlations between FC and CDAI.

Several studies have shown that faecal MPO concentrations also correlate with disease severity (24, 25, 61, 62). Saiki et al. (25) found that MPO is present in low amounts in the stool of patients with inactive IBD and normal controls, and that it markedly increases in patients with active disease. A paired analysis of the same individual also showed a decrease in MPO concentrations after the resolution of disease exacerbation. Daily variations of MPO concentrations during the course of the
disease further supported the reliability of this data. The author concluded that MPO measurement may help determine disease prognosis and confirm treatment efficacy.

Masoodi et al. (61) demonstrated that increasing endoscopic severity was associated with higher MPO concentrations, but this did not reach statistical significance due to the small number of patients in each subgroup. A significant reduction in faecal MPO was observed after treatment, which correlated with simultaneous reduction in endoscopic severity. MPO was significantly higher in patients with idiopathic ulcerative colitis (IUC) than in controls. This suggested that faecal MPO is a sensitive marker for the presence of colonic inflammation with sensitivity of 89%. However, MPO has a specificity of 51.4% as 48% of controls were MPO positive. It is possible that these controls may have had low-grade asymptomatic colonic inflammation.

Sugi et al. (24) found that MPO concentrations were significantly increased in active IBD compared to inactive disease in both UC and CD. Similarly, Peterson et al. (62) found that faecal MPO was elevated in all patients with active IBD, and striking reductions in faecal concentrations of MPO were observed after four weeks of treatment in 20/28 patients with complete remission after eight weeks. Concentrations of faecal MPO also related to histological indices of disease.

The literature is mixed with respect to the most appropriate cut-off value when measuring disease activity. A recent study indicated that a cut-off point of 50.0 μg/g for calprotectin had a 91.9% sensitivity and a 79.4% specificity for making a differentiation between active UC and inactive UC (50). Another study used a cut-off of 100 μg/g for calprotectin which gave a sensitivity of 80% and specificity 67% when identifying those with or without inflammation (59). Two other studies have assessed the potential use of FC with clinical indices. The first study (63), which applied a cut-off of 150 μg/g, showed a sensitivity and specificity of 89% and 82% respectively in UC patients, and of 87% and 43% respectively in CD patients. The second study (64), using a cut-off 250 μg/g, showed a sensitivity and specificity of 90% and 83% respectively for the whole group of IBD patients. However, Walkiewicz et al. (49) found that an FC value of 400 μg/g was most appropriate. A specific cut-off value was rarely stated for MPO throughout the literature, though one study stated that levels greater than, or equal to 0.065 units/ml was considered positive (61). A variety of calprotectin cut-off values used for measuring disease severity are summarised in the table below.
Table 1.1 Calprotectin cut-off values (µg protein/g faeces) for measuring disease severity

<table>
<thead>
<tr>
<th>Study</th>
<th>Cut-off values (µg/g)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xiang et al.</td>
<td>50</td>
<td>92</td>
<td>79</td>
<td>Active and inactive UC</td>
</tr>
<tr>
<td>Gaya et al.</td>
<td>100</td>
<td>80</td>
<td>67</td>
<td>With or without inflammation</td>
</tr>
<tr>
<td>Tibble et al.</td>
<td>150</td>
<td>89</td>
<td>82</td>
<td>Inflammation in UC patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87</td>
<td>43</td>
<td>Inflammation in CD patients</td>
</tr>
<tr>
<td>Berni Canani et al.</td>
<td>250</td>
<td>90</td>
<td>83</td>
<td>Inflammation in IBD patients</td>
</tr>
</tbody>
</table>

1.8 Predicting relapse

IBD has a typically relapsing course; therefore, the main goal of treatment of IBD is effective and lasting suppression of the inflammatory response in order to induce and maintain clinical remission. Nonetheless, even in cases of successful treatment, subclinical inflammation of the intestinal wall may persist, contributing significantly to the risk of relapses (65). Because the timing of relapses can be unpredictable, it makes sense that clinical symptoms rather than invasive tests are used to monitor disease activity. However, most IBD relapses come to medical attention after the inflammatory response has become well-established and clinical symptoms are worsening. The use of calprotectin as a predictor of relapse in patients with quiescent IBD has been examined in several studies.

Tibble et al. (63) studied 43 patients with CD and 37 patients with UC in clinical remission for 1-4 months as determined by clinical disease activity indices and the HBI. These patients had calprotectin concentrations at baseline and were then followed for 1 year. Approximately half of the patients in each group relapsed during the 12 month follow up period. The sensitivity and specificity of calprotectin for predicting relapse within the year in all patients with IBD were 90% and 83% respectively, using a cut-off of 50 mg/L. This cut off was associated with a 13-fold increased risk of relapse. These values were similar when patients with CD or UC were examined separately.

Costa et al. (48), found nearly identical sensitivity and specificity of calprotectin for predicting relapse in UC, but their specificity of calprotectin in CD was 43%, markedly lower than the 83% reported by Tibble et al (63). This large discrepancy may be explained by the fact that their study used different assays and a lower cut off FC (150 µg/g, corresponding to 30 mg/L).
A more recent study prospectively evaluated 97 patients with UC and 65 with CD in clinical remission for at least 6 months (66). The cut off level was set at 130 mg/kg of faeces. Patients were followed up for 1 year after the test or until relapse. FC was positive in 44 UC patients and 26 of them relapsed within a year, while 11 of 53 UC patients with a negative calprotectin relapsed within the year. Thirty CD patients had a positive calprotectin and 13 of them relapsed within a year, as did 7 of the 35 with a negative test result. A significant correlation emerged between a positive calprotectin and the probability of relapse in UC patients. In CD patients, only cases of colonic CD showed a significant correlation between a positive calprotectin test and the probability of relapse, such that 6 colonic CD patients were positive for the calprotectin test and 4 relapsed.

As previously shown in adults (55, 67-69), FC concentrations are higher in children with IBD compared to controls regardless of disease activity (5, 64, 70, 71). In addition, FC was significantly elevated in children with IBD who were in relapse compared to those who were in clinical remission (64, 70). Walkiewicz et al. (49) conducted a study to compare FC concentrations in IBD and healthy controls, to correlate FC concentrations with disease activity, and to assess whether FC concentrations can be used to predict clinical relapse in children with IBD. The authors concluded that FC values of 400 µg/g or more may predict an impending clinical relapse within the next 9 months in patients with quiescent CD. Conversely, since 89% of CD patients with FC concentrations of less than 400 µg/g stayed in remission, this cut off value may predict those who will stay in clinical remission.

Similarly, Diamanti et al. (6) assessed the clinical efficacy of the calprotectin assay in determining histological relapses of paediatric IBD patients. This study validated the measurement of FC as a sensitive and specific measure of histological relapse in IBD children. The 36 months of histological follow up demonstrated that in patients with quiescent IBD, FC was useful in determining histological relapses.

### 1.9 Treatment outcome

Recent studies and reviews suggest mucosal healing is becoming a therapeutic target for treatment of IBD (72-75). During infliximab treatment, clinical improvement is accompanied by significant healing of endoscopic lesions and the disappearance of mucosal inflammatory infiltrate (76). Several studies have shown that the use of faecal markers in assessing the treatment outcome of patients with IBD was promising. Sipponen et al. (77) compared FC and lactoferrin to the Crohn's disease endoscopic index of severity (CDEIS), to the CDAI, and to histological activity before and after anti-TNF-α therapy. The results showed that both FC and lactoferrin correlated closely with endoscopic activity during anti-TNF-α therapy, and that their elevated concentration was a highly specific surrogate marker of endoscopically active disease. The concentration of calprotectin and lactoferrin in stool declined in treatment responders significantly from the baseline level and normalised in almost
all those who reached endoscopic remission. The authors concluded that these two markers serve well as surrogate markers for mucosal healing.

Similarly, Roseth et al. (46) used infliximab treatment to treat patients with severe relapsing CD and found that after treatment, over a period of 3-4 weeks, FC values decreased from 1000-2000 mg/L to 200-300 mg/L, with higher values often correlating with clinically active disease (78). They also found that FC concentrations were normalised after treatment, based on both an endoscopically and histologically normal mucosal lining; furthermore, the prognosis of patients with mucosal healing was better compared to non-healing patients. Therefore, the authors suggested that mucosal healing should be a treatment goal for IBD patients, and that FC is a significant marker in determining disease activity and response to treatment in IBD patients.

Masoodi et al. (61) examined the role of serum C-reactive protein (SCRP), MPO and faecal lactoferrin in assessing disease severity, activity and response to therapy. Markedly elevated levels of MPO, faecal lactoferrin titers and CRP-positivity indicated the presence of more severe disease. Following treatment, all patients had significant clinical improvement, and all three biomarkers studied showed a significant fall at follow up. Sangfelt et al. (26) also observed similar results when they studied rectal perfusate before and during prednisone enema treatment. They observed a significant decrease in MPO concentrations if the patient responded to treatment. This was seen more often before clinical improvement. Thus rectally released MPO seems to be an early marker of mucosal healing and treatment response in IUC patients. Markedly elevated concentrations of MPO indicated the presence of active disease, and the significant fall in concentrations at follow up were concluded to reflect mucosal healing and clinical remission.

Another recent study (7) evaluated FC as a surrogate marker of treatment outcome of relapse of IBD and compared FC with MPO and faecal eosinophil protein X (EPX). A close correlation between FC, MPO and EPX and treatment outcome was observed, and FC and MPO were found to provide better assessment of treatment outcome compared to EXP in patients with UC.

1.10 Conclusions and research objectives

IBD is a relapsing-remitting condition, and so far, a majority of studies have shown that the best way to assess inflammation is through colonoscopy with biopsy; however, this is an invasive method and carries risks of complications (44). Patients who present with chronic diarrhoea represent a wide range of diagnoses which have very different prognoses and treatment. There is a growing body of evidence that faecal markers are promising and may be more specific in detecting gut inflammation in patients with IBD. The potential of faecal markers to identify patients with IBD, monitor their treatment outcomes, and to assess their risk of relapse is an appealing prospect. Gastroenterologists would
therefore be able to diagnose IBD at a much faster rate by eliminating the wait time for a colonoscopy. In addition, they would be able to individualise treatment by prescribing more powerful drugs to patients at risk of relapse, while patients at reduced risk of relapse would avoid these more powerful drugs.

The ideal faecal marker of inflammation would have high sensitivity to the presence of inflammation, be stable for long periods of time allowing for transport to laboratories, and be validated on small samples as well as in multiple day homogenised collections. It is also important that the faecal marker is not interfered with by diet or pharmacotherapy, and is specific to the cause of inflammation. From the literature it can be seen that the ability of the faecal marker calprotectin in the diagnosis and follow up of IBD has been extensively studied; however, there are still mixed views on the accuracy of this marker in various diagnostic situations. The majority of studies conclude the simplicity of the calprotectin assay, its relatively low cost and its sensitivity suggests that it has potential as a routine screening test; however, currently it should still be used in conjunction with endoscopy with biopsy for making a definitive diagnosis of IBD. The literature assessing the ability of the faecal marker myeloperoxidase in diagnosis and follow up of IBD is limited; nonetheless, the results have also been promising.

In the current study, we hypothesise that calprotectin and MPO in faecal samples will correlate strongly with disease severity in IBD, and aim to assess whether MPO is a superior marker to calprotectin. Calprotectin will be measured by ELISA, and MPO will be measured by ELISA and a simple peroxidase assay.
2 Methods

2.1 Reagents and Equipment

The name and location of manufacturing of all specialist reagents and equipment are indicated here, and subsequently referred to by suppliers name only in the first instance of this chapter.

The products used in this research were from various suppliers. However the rabbit anti-MPO polyclonal antibody used in the ELISA assay was produced in house. All reagents not in the list below were purchased from Biolab thermofisher chemicals in Australia.

**Costar, USA**
- 96 well flat bottom ELISA plate

**Dako, Denmark**
- Biotinylated polyclonal anti-MPO antibody

**Invitrogen, NZ**
- AmplexRed
  - Bovine Serum Albumin (product code = 30060-578)

**J.T. Baker, USA**
- Dimethylformamide (DMF)

**Labserv, Australia**
- Hydrogen Peroxide (product code = BSPA5.500)

**Sigma, USA**
- Tween-20
  - Extra-Avidin alkaline phosphatase (product code = E2636.5ML)
  - Tetramethylbenzidine (TMB)

**Strem chemicals, USA**
- P-nitrophenyl phosphate disodium salt (PNP)

**Vital diagnostics, Australia**
- Phical calprotectin ELISA kit
2.2 Recruitment of patients and samples

Patients coming into Christchurch Hospital to have a colonoscopy that had a previous diagnosis of IBD, or who were suspected of having IBD were recruited into the Evaluation of Novel Biomarkers for Inflammatory Bowel Disease study (NBIBD). Initial stool, blood and biopsy samples were taken when the patients came in for their procedure. They also filled out a questionnaire based on their symptoms prior to the procedure. A nine month follow up was then carried out, and every three months for the next nine months patients were required to send in another stool sample, blood sample, and symptoms questionnaire. This meant that samples were collected from patients on four separate occasions over the period of a year. Samples were aliquoted out and stored in either -20 or -80°C freezers. For this project 100 of the initial stool samples that had been stored at -80°C were analysed. Of these 100 samples 20 were controls (patients with no IBD), 42 were patients with CD, and 38 were patients with UC. The patients with IBD had varying disease severity.

2.3 Disease severity scores

2.3.1 Clinical disease activity scores

2.3.1.1 Harvey-Bradshaw Index

The Harvey-Bradshaw index (HBI) is used to quantify the symptoms of patients with CD. This index consists of only clinical parameters:

1. general well-being (0 = very well, 1 = slightly below average, 2 = poor, 3 = very poor, 4 = terrible)
2. abdominal pain (0 = none, 1 = mild, 2 = moderate, 3 = severe)
3. number of liquid stools per day
4. abdominal mass (0 = none, 1 = dubious, 2 = definite, 3 = tender)
5. complications, as above, with one point for each

The total score is calculated from the individual score received with respect to each of the five parameters. Patients with CD who scored 3 or less on the HBI are likely to be in remission; whereas, patients with a score of 8 to 9 or higher are considered to have severe disease. This questionnaire was completed by patients approximately 24 hours prior to coming in for colonoscopy and was collected on the day of the procedure.
2.3.1.2 Simple Clinical Colitis Activity Index

The simple clinical colitis activity index (SCCAI) is used to quantify the symptoms of patients with UC. This questionnaire was completed by patients approximately 24 hours prior to coming in for colonoscopy and was collected on the day of the procedure. Table 2.1 consists of the clinical parameters used to assess UC disease activity. The patient will circle the number of bowel motions they have per day and night, how urgently they need to pass a bowel motion, how much blood is in their stool, and their general wellbeing. The corresponding score to the right is given, and the total is calculated.

Table 2.1. Simple clinical colitis activity index

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bowel frequency (day)</td>
<td></td>
</tr>
<tr>
<td>1–3</td>
<td>0</td>
</tr>
<tr>
<td>4–6</td>
<td>1</td>
</tr>
<tr>
<td>7–9</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 9</td>
<td>3</td>
</tr>
<tr>
<td>Bowel frequency (night)</td>
<td></td>
</tr>
<tr>
<td>1–3</td>
<td>1</td>
</tr>
<tr>
<td>4–6</td>
<td>2</td>
</tr>
<tr>
<td>Urgency of defecation</td>
<td></td>
</tr>
<tr>
<td>Hurry</td>
<td>1</td>
</tr>
<tr>
<td>Immediately</td>
<td>2</td>
</tr>
<tr>
<td>Incontinence</td>
<td>3</td>
</tr>
<tr>
<td>Blood in stool</td>
<td></td>
</tr>
<tr>
<td>Trace</td>
<td>1</td>
</tr>
<tr>
<td>Occasionally frank</td>
<td>2</td>
</tr>
<tr>
<td>Usually frank</td>
<td>3</td>
</tr>
<tr>
<td>General well being</td>
<td></td>
</tr>
<tr>
<td>Very well</td>
<td>0</td>
</tr>
<tr>
<td>Slightly below par</td>
<td>1</td>
</tr>
<tr>
<td>Poor</td>
<td>2</td>
</tr>
<tr>
<td>Very poor</td>
<td>3</td>
</tr>
<tr>
<td>Terrible</td>
<td>4</td>
</tr>
<tr>
<td>Extracolonic features</td>
<td></td>
</tr>
<tr>
<td>1 per manifestation</td>
<td></td>
</tr>
</tbody>
</table>

2.3.2 Endoscopy scores

2.3.2.1 Simple Endoscopic Score for Crohn’s Disease

For calculating the Simple Endoscopic Score for Crohn’s Disease (SES-CD), the intestine was divided into five segments: the ileum, the right colon, the transverse colon, the left colon and the rectum. The degree of disease involvement in each of the five segments was determined by the assessment of four parameters: presence and size of ulcers (score 0-3), extent of ulcerated surface (score 0-3), extent of affected surface (score 0-3), and presence and type of narrowing (score 0-3). The sum of the score for each endoscopic variable ranges from 0 to 15, except for stenosis, where it varies between 0 and 11, because 3 represents a stenosis through which a colonoscope cannot be
passed, and therefore can be observed only once. The lowest possible SES-CD was 0, representing an intestine without any lesions; the highest possible score was 56 points. SES-CD activity levels were defined as follows: inactive (remission) 0-3, mild activity 4-10, moderate activity 11-19, and high activity ≥20 points. The criteria for assessing SES-CD is summarised in Table 2.2.

Table 2.2. Definitions of the Simple Endoscopic Score for Crohn’s Disease (SES-CD) variables

<table>
<thead>
<tr>
<th>SES-CD score</th>
<th>Variable</th>
<th>Presence of ulcers</th>
<th>Ulcerated surface</th>
<th>Affected surface</th>
<th>Presence of narrowings</th>
<th>Number of affected segments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Unaffected surface</td>
<td>None</td>
<td>All variables = 0</td>
</tr>
<tr>
<td>1</td>
<td>0.1-0.5 cm</td>
<td>Aphtous ulcers</td>
<td>&lt; 10%</td>
<td>&lt; 50%</td>
<td>Single, can be passed</td>
<td>At least one variable ≥ 1</td>
</tr>
<tr>
<td>2</td>
<td>0.5-2 cm</td>
<td>Large ulcers</td>
<td>10-30%</td>
<td>50-75%</td>
<td>Multiple, can be passed</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&gt; 2 cm</td>
<td>Very larger ulcers</td>
<td>&gt; 30%</td>
<td>&gt; 75%</td>
<td>Cannot pass</td>
<td></td>
</tr>
</tbody>
</table>

2.3.2.2 Rachmilewitz Score for Ulcerative colitis

For calculating the Rachmilewitz score four items were included: granulation scattering reflected light (score 0-2), vascular pattern (score 0-2), vulnerability of mucosa (score 0-4), and mucosal damage (mucus, fibrin, exudates, erosions, and ulcers, score 0-4). The sum of the score for each endoscopic variable ranges from 0 to 12. The lowest possible Rachmilewitz score was 0 and the highest possible score was 17. Rachmilewitz activity levels were as follows: inactive (0-3), mild (4-6), moderate (7-9), and high activity (10-12). The criteria for assessing the Rachmilewitz score is summarised in Table 2.3.

Table 2.3. Definitions of the Rachmilewitz score for Ulcerative colitis variables

<table>
<thead>
<tr>
<th>Endoscopic Activity Index</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.Granulation scattering reflected light</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>2.Vascular pattern:</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Faded/disturbed</td>
<td>1</td>
</tr>
<tr>
<td>Completely absent</td>
<td>2</td>
</tr>
<tr>
<td>3.Vulnerability of mucosa</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Slightly increased (contact bleeding)</td>
<td>2</td>
</tr>
<tr>
<td>Greatly increased (spontaneous bleeding)</td>
<td>4</td>
</tr>
<tr>
<td>Mucosal damage (mucus, fibrin, exudates, erosions, ulcer):</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Slight</td>
<td>2</td>
</tr>
<tr>
<td>Pronounced</td>
<td>4</td>
</tr>
</tbody>
</table>
A specialist Gastroenterologist determined the endoscopy scores for the patients with IBD.

2.4 Specimens

Collect 1-5 grams of faeces and place into suitable container. During transportation of samples to the laboratory, the samples should not exceed temperatures of 30°C. Store the samples at -80°C until time of analysis. Before the extraction samples were thawed out overnight in a refrigerator.

2.5 Preparation of faecal samples

Approximately 100 mg of the faecal sample was placed into the bottom cap of the extraction tube. Taking any solid, undigested material like fibres and seeds was avoided. The tube containing the faecal sample was weighed, and this weight was recorded. The working solution of extraction buffer was added with a weight to volume ratio of 1:50 was added, i.e. 4.9 mL to 100 mg faeces (see table below). The extraction tube containing extraction buffer and the faecal sample was vortexed vigorously for 30 seconds. Mixing was continued on Chiltern rollers for a further 10 minutes. Extraction tubes were then centrifuged for 10 minutes at 3000 rpm and the supernatant was then ready to be used in the assay.

Table 2.4 Weights and dilutions

<table>
<thead>
<tr>
<th>Stool (mg)</th>
<th>Extraction solution (mL)</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
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<td>3.2</td>
</tr>
<tr>
<td>60</td>
<td>2.9</td>
</tr>
</tbody>
</table>
2.6 Calprotectin assay

The CALPRO Calprotectin ELISA test is based upon preparation of an extract of about 100 mg of faeces mixed with about 5 mL of faecal extraction buffer in a closed tube. After centrifugation, a sample from the supernatant is tested by an enzyme-linked immunosorbent assay specific for calprotectin.

The immunoassay requires that samples and standards are incubated in separate microtiter wells coated with polyclonal antibodies against calprotectin. After incubation and washing of the wells, bound calprotectin is allowed to react with immunoaffinity-purified enzyme-labelled anti-calprotectin antibodies. Thus the amount of enzyme bound is roughly proportional to the amount of calprotectin in the sample or standard, which is determined by incubation with a substrate for the enzyme.

The rabbit antibodies used in the CALPRO Calprotectin ELISA Test react with a number of different epitopes on calprotectin, ensuring a positive signal even if some epitopes are damaged or hidden due to complex formation with other substances in the stool. The CALPRO Calprotectin ELISA Test is run on stool extracts prepared by the use of patented-faecal extraction buffer which brings calprotectin into solution in a molecular configuration like that in leukocyte extracts or plasma. This is important because quantitative immunoassays require that proteins in the standards and samples have the same configuration.

2.6.1 Faecal extraction

Calprotectin was extracted from the 100 faecal samples using the procedure stated in section 2.5. The extraction buffer used was supplied in the Phical Calprotectin ELISA kit. Ordered via Vital Diagnostics, NSW, Australia.

2.6.2 Procedure

All standards and samples were carried out in duplicate. Samples and pooled quality control (QC) were diluted 1:50 (20 μL sample and 980 μL of dilution buffer). Standards and controls were ready to use. 50 μL of standard zero (dilution buffer) was pipetted into wells A1 and B1. 50 μL of the remaining standards and controls were pipetted into the appropriate wells. 50 μL of the diluted pool control and patient samples were pipetted into the appropriate wells. The plate was then covered with sealing foil and incubated at room temperature on the MSI mini-shaker for 45 minutes. The wells were then washed using the ELISA automatic washing apparatus. After the final aspiration the plate was inverted and tapped gently on a towel or absorbent tissue to remove any remaining washing solution. Using a multi-channel pipette 50 μL of conjugate was added to each well. The plate was then covered with sealing foil and placed back onto the mini-shaker for another 45 minute incubation.
washing step was then repeated. Using a multi-channel pipette, 100 μL of substrate solution was added to each well. The plate was then incubated for 30 minutes at room temperature in the dark. An ELISA plate reader was then used to read the OD values under the calprotectin format.

2.7 Myeloperoxidase assay

This assay measures both MPO protein and MPO activity. The specific activity of MPO was calculated from the ratio of these two measurements. MPO is bound via a monoclonal antibody to an Enzyme-linked Immunosorbent Assay (ELISA) plate and its activity measured using a substrate that gives a fluorescent product. The plate is then washed and a polyclonal anti-MPO antibody is used to determine the amount of MPO protein bound to the plate.

2.7.1 Faecal extraction

MPO was extracted from the 100 faecal samples using the procedure stated in section 2.5. The extraction buffer used was 500 mM NaCl in PBS.

2.7.2 Procedure

An ELISA for MPO was developed using high binding flat bottom 96 well immunoplates (Costar, USA). Plates were coated overnight at room temperature, or for 2 hours at 37°C with 50 μL/well of mouse monoclonal antibody to human MPO (Abcam, AB10165 [4A4]) and diluted 1/1200 in PBS. After washing the plate with PBS, additional protein binding sites were blocked by incubation with 75 μL/well of assay buffer (1% BSA, 0.025% Tween-20 in PBS) for 1-2 hours at room temperature. After washing the plate with PBS, 50 μL/well of samples and standards (MPO protein ranging from 0-50 μL/mL or from 0-340 pM) were then added and incubated for 1 hour at 37°C.

2.7.2.1 Measuring myeloperoxidase activity

After washing the plate with PBS, MPO activity was measured by adding 50 μL/well of fluorescent substrate (a mixture containing 50 μM AmplexRed, 20 μM hydrogen peroxide and 50 mM bromide in 50 mM Phosphate buffer pH - 7.4). Production of the fluorescence product was measured using λex 544 nm λem 590 nm over 10 minutes. A standard curve was then plotted for the fluorescence changes at 5 minutes versus the concentration of MPO. The MPO activity in the samples was determined using the standard curve.
2.7.2.2 Measuring myeloperoxidase protein

After washing the plate with PBS, 50 µL/well of an in house rabbit polyclonal anti-MPO serum diluted 1/800 in assay buffer was added and incubated for 1 hour at 37°C. The anti-MPO serum was produced by the Free Radical Research Group but could be replaced by a commercial polyclonal anti-MPO antibody. After washing the plate with PBS, 50 µL/well of biotinylated polyclonal goat anti-rabbit antibody (Dako) diluted 1/2000 in assay buffer was added and incubated for 1 hour at 37°C. The wash step was then repeated and 50 µL/well of Extra/Avidin Alkaline Phosphatase (Sigma) diluted 1/1000 in assay buffer was added and incubated for 1 hour at room temperature. After washing the plate again with PBS, 50 µL/well of substrate (1 mg/ml p-nitrophenyl phosphate disodium salt in 10% diethanolamine with 0.5 mM MgCl₂) was added and incubated for 30 minutes at room temperature followed by 15 minutes at 37°C. The production of yellow chromophore was measured at 405 nm. A standard curve was plotted for absorbance versus the concentration of MPO protein in the standards. The unknown MPO protein content in the samples was determined using the standard curve.

2.7.3 Myeloperoxidase recovery

Three extraction buffers (PBS + 500 mM NaCl, PBS + 0.03% cetrimide and PBS + 500 mM NaCl + 0.03% cetrimide) were compared and MPO activity and protein were measured using ELISA. 100 mg of a stool sample containing no MPO was aliquot into six Eppendorf tubes. Three out of the six were spiked with 200 μL of 500 nM MPO, and 200 μL of 1% cetrimide + PBS buffer was added to the other three Eppendorf tubes. All samples were homogenised, vortex for 30 seconds, sonicated for 1 minute and then placed on a vortex shaker for a further 45 minutes. Each sample was then transferred to a separate 14 mL tube and 4.9 mL of one of the three extraction buffers was added to each of the three tubes. The same process was followed for the three tubes with 0 nM MPO. Contents of the tubes were mixed for 20 minutes and then centrifuged for 10 minutes at 3000 rpm. The tubes were stored in the cold room (4°C) overnight. All standards and samples were carried out in duplicate. The samples were diluted 1:10 (20 µL sample and 180 µL ELISA buffer), and standards ranged from 1 nM MPO to 0 nM MPO. The next day ELISA was run on each of the samples following the procedure from section 2.7.2 and results were obtained.
2.8 Tetramethylbenzidine assay

3,3′,5,5′-tetramethylbenzidine (TMB) is a chromogenic substrate which is used as a visualising reagent. In solution, TMB forms a blue product when it reacts with peroxidase enzymes such as MPO. The resulting colour change can be read on a microplate reader at a wavelength of 630 nm. The reaction can be halted by addition of acid or another stop reagent.

The MPO standards ranged from 1 nM to 0 nM MPO and standards were diluted in acetate buffer + 0.01% cetac. Patient samples were diluted in acetate buffer alone.

The TMB reagent was made up as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (µL)</th>
<th>Final Concentration (mM)</th>
</tr>
</thead>
<tbody>
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<td>5 mM TMB</td>
<td>1500</td>
<td>2.4</td>
</tr>
<tr>
<td>1 M Br</td>
<td>240</td>
<td>20</td>
</tr>
<tr>
<td>90 mM H$_2$O$_2$</td>
<td>39</td>
<td>0.3</td>
</tr>
<tr>
<td>300 mM NaAc</td>
<td>4221</td>
<td>150</td>
</tr>
</tbody>
</table>

300 mM Sodium acetate pH 5.4 (NaAc) was added to the TMB first as this maintains the high DMF concentration and avoids TMB precipitation. 90 mM hydrogen peroxide (H$_2$O$_2$) was added 5 minutes after mixing the TMB with NaAc as the reaction is exothermic. 100 µL of supernatant was added to the 96 well microplate, then 100 µL of TMB reagent was added to each well. OD$_{630}$ values were then read from the Dynatech microplate reader straight after the TMB reagent was added, and at 5 and 10 minutes.

2.8.1 Extraction buffers

Three extraction buffers (PBS, PBS + 0.01% cetac and PBS + 500 mM NaCl) were compared and the activity of MPO measured using TMB as the peroxidase substrate. MPO was extracted from ten samples with suspected high disease activity (due to the patients symptoms at the time of sample collection) using the procedure stated in section 2.5. All of the samples were extracted three times, each time using a different extraction buffer; PBS, PBS + 0.01% cetac and PBS + 500 mM NaCl. All standards and samples were carried out in duplicate. Samples were diluted 1:20 (20 µL sample and 380 µL of acetate buffer), and the standards ranged from 1 nM MPO to 0 nM MPO. 100 µL of standard 0 (acetate buffer + 0.01% cetac) was pipetted into wells A1 and A2. 100 µL of the remaining standards and patient samples were pipetted into the appropriate wells. 100 µL of the TMB reagent stated in section 2.8 was added to each well giving the patient samples a total dilution of 1:40. OD$_{630}$
values were then read from the Dynatech microplate reader straight after the TMB reagent was added, and at 5 and 10 minutes.

2.8.2 Inhibition of myeloperoxidase

The detection of MPO activity was confirmed by TMB assay using a specific inhibition of the enzyme by 2-thioxanthine (79) and including bromide to enhance its activity. The MPO from three samples with suspected high disease activity due to the patient’s symptoms at the time of sample collection was extracted using PBS + 500 mM NaCl. The positive control 1 nM MPO was also used. All standards, samples and the positive control were carried out in duplicate. Samples were diluted 1:5 (60 μL sample and 240 μL acetate buffer) and standards ranged from 1 nM MPO to 0 nM MPO. 100 μL of standard 0 (acetate buffer + 0.01% cetac) was pipetted into wells A1 and A2. 100 μL of the remaining standards and patient samples were pipetted into the appropriate wells. Three different treatments were applied to each of the samples and the 1 nM MPO positive control. The first treatment applied was the original TMB reagent as stated in section 2.8 without bromide. The second treatment applied was the original TMB reagent as stated in section 2.8, and the third treatment applied was the original TMB reagent with 2-thioxanthine (the specific MPO inhibitor) added. 5 μL of 10 mM 2-thioxanthine was added to 495 μL acetate buffer to give a concentration of 0.1 mM. 200 μL of 0.1 mM 2-thioxanthine was added to 980 μL of the TMB reagent (250 μL 10 mM TMB, 40 μL 1M bromide, 6.5 μL 90 mM H₂O₂, and 503.5 μL acetate buffer). These reagents had final concentrations of 2.4 mM TMB, 20 mM bromide, 0.3 mM H₂O₂, 150 mM NaAc, and 10 μM 2-thioxanthine on the plate. 100 μL of standard 0 (acetate buffer + 0.01% cetac) was pipetted into wells A1 and A2. 100 μL of the remaining standards and patient samples were pipetted into the appropriate wells. 100 μL of the appropriate treatment was added to each well giving the patient samples a total dilution of 1:10. OD₆₃₀ values were then read from the Dynatech microplate reader straight after the TMB reagent was added, and at 5 and 10 minutes.

2.8.3 Myeloperoxidase distribution

MPO was measured by ELISA in four different parts of each faecal sample, and concentrations were compared to assess within sample variation. 100 mg of stool was taken from four different areas within the sample (side, top, middle and bottom of the sample). A total of eight samples with suspected high disease activity due to the patient’s symptoms at the time of sample collection were used. MPO was extracted from these samples using the procedure stated in section 2.5, and the extraction buffer used was 500 mM NaCl in PBS. All standards and samples were carried out in duplicate. All samples were diluted 1:10 (20 μL sample and 180 μL assay buffer), and standards ranged from 1 nM MPO to 0 nM MPO. The MPO ELISA protocol was followed from section 2.7.2 and results were obtained.
3 Results

3.1 Introduction

This section describes the development and optimisation of the MPO ELISA assay which includes the assessment of the most effective extraction buffer, MPO recovery, specific MPO inhibition and MPO distribution throughout the stool sample. The levels of the two faecal markers calprotectin and MPO were (1) correlated against each other, (2) compared between the control, CD and UC groups, (3) correlated with disease activity, (4) correlated with endoscopic indices and (5) compared between disease severity groups based on their endoscopy scores.
3.2 Myeloperoxidase assay development and optimisation

3.2.1 Extraction buffers

Three different extraction buffers were used to extract MPO from 10 pilot samples with expected high levels of inflammation due to the patient’s symptoms at the time of sample collection. Three extraction buffers used were PBS, PBS + 0.01% cetac, and PBS + 500 mM NaCl, and the amount of MPO extracted from the samples was measured using the MPO TMB assay. Unfortunately, only two out of the 10 samples collected had detectable levels of MPO. The third extraction buffer PBS + 500 mM NaCl was the most effective at extracting the MPO from both of the samples (Figure 3.1). However, there was no significant difference between the three extraction buffers (p = 0.333) when the Kruskal-Wallis one way analysis of variance on ranks was used to analyse the data.

![Figure 3.1](image)

**Figure 3.1.** The three extraction buffers (PBS, PBS + 0.01% cetac, and PBS + 500 mM NaCl) were used to extract the MPO from the two samples. (A) Sample one MPO TMB activity levels in the extracts were 55.57 ± 2.09 μg protein/g faeces for PBS, 61.17 ± 4 μg protein/g faeces for PBS + 0.01% cetac, and 67.69 ± 3.32 μg protein/g faeces for PBS + 500 mM NaCl. (B) Sample two MPO TMB activity levels in the extracts were 51.93 ± 1.78 μg protein/g faeces for PBS, 32.27 ± 0.11 μg protein/g faeces for PBS + 0.01% cetac, and 70.5 ± 4.36 μg protein/g faeces for PBS + 500 mM NaCl.
3.2.2 Myeloperoxidase recovery

One control sample with no detectable MPO was used for the MPO recovery experiment. The sample was split into six aliquots. Three out of the six were spiked with 200 μL of 500 nM MPO and the other three were spiked with 200 μL of 1% cetrimide + PBS buffer. Three extraction buffers were used to extract the MPO from the 0 nM MPO and 500 nM MPO spiked samples. The three extraction buffers used were PBS + 500 mM NaCl, PBS + 0.03% cetrimide, and PBS + 0.03% cetrimide + 500 mM NaCl, and the amount of MPO extracted from the sample was measured using ELISA. The most effective extraction buffer for extracting MPO from the 500 nM MPO spiked stool samples was PBS + 500 mM NaCl (Figure 3.2). This result was consistent with the results obtained from section 3.2.1. When the PBS + 500 mM NaCl extraction buffer was used 24% of the MPO protein was recovered. 14% of the MPO protein was recovered when the PBS + 0.03% cetrimide buffer was used, and only 9% of the MPO protein was recovered when the PBS + 0.03% cetrimide + 500 mM NaCl extraction buffer was used. As expected no MPO was detected in the 0 nM spiked MPO samples.

![Figure 3.2](image-url)

**Figure 3.2.** Three extraction buffers (PBS + 500 mM NaCl, PBS + 0.03% cetrimide, and PBS + 0.03% cetrimide + 500 mM NaCl) were used to extract the MPO from the 500 nM MPO spiked MPO sample. The MPO protein levels in the spiked 500 nM MPO were 23.64 ± 6.74 μg protein/g faeces for PBS + 500 mM NaCl, 12.76 ± 6.419 μg protein/g faeces for PBS + 0.03% cetrimide, and 8.8 ± 0.71 μg protein/g faeces for PBS + 0.03% cetrimide + 500 mM NaCl.
3.2.3 Inhibition of myeloperoxidase

The detection of MPO activity by TMB assay was confirmed using the specific MPO inhibitor 2-thioxanthine \((79)\), and including bromide to enhance its activity. Three pilot samples with suspected high levels of MPO TMB activity due to the patient’s symptoms at the time of sample collection were used. A 1 nM MPO positive control was also used. Three treatments were applied, the original TMB reagent without bromide as stated in section 2.8, the original TMB reagent with bromide, and the original TMB reagent with 10 μM 2-thioxanthine. Two out of the three pilot samples had detectable levels of MPO TMB activity and could therefore be used. The presence of 2-thioxanthine and the absence of bromide successfully decreased the MPO TMB activity in all of the samples (Figure 3.3). The presence of 2-thioxanthine decreased the MPO TMB activity in the positive control from 77.35 μg protein/g faeces to 21.41 μg protein/g faeces (a 72% decrease), and in the absence of bromide MPO TMB activity decreased from 77.35 μg protein/g faeces to 27.75 μg protein/g faeces (a 64% decrease). The presence of 2-thioxanthine decreased the MPO TMB activity of sample 1 from 12.62 μg protein/g faeces to 5.858 μg protein/g faeces (a 54% decrease), and in the absence of bromide MPO TMB activity decreased from 12.62 μg protein/g faeces to 5.78 μg protein/g faeces (a 54% decrease). The presence of 2-thioxanthine decreased the MPO TMB activity of sample 2 from 75.2 μg protein/g faeces to 22.87 μg protein/g faeces (a 70% decrease), and in the absence of bromide MPO TMB activity decreased from 75.2 μg protein/g faeces to 52.7 μg protein/g faeces (a 30% decrease).

The bromide effect suggests that MPO may not be the only peroxidase present, as the activity was expected to be lower when no bromide was added to the TMB reagent. However, the activity was reduced more substantially in the presence of 2-thioxanthine indicating that the activity detected in the samples was predominantly coming from MPO.
Figure 3.3. Three treatments (original TMB reagent without bromide, with bromide, and with 2-thioxanthine) were applied to the two pilot samples and one positive control. The presence of 2-thioxanthine and the absence of bromide successfully decreased the MPO TMB activity in all of the samples.
3.2.4 Myeloperoxidase distribution

MPO protein was measured by ELISA in four different parts of each of the pilot samples with suspected high MPO protein levels due to the patient’s symptoms at the time of sample collection. Eight pilot samples were collected; however, only three out of the eight had detectable MPO protein levels. MPO protein levels appear to be varied across each part of the faecal sample. However, when the data is normalised and analysis of variance is calculated it indicates that there is no significant difference between each of the areas within the stool samples (p = 0.156). Figure 3.4 illustrates the levels of MPO protein in four areas within each of the stool samples.

![Myeloperoxidase protein levels](image)

**Figure 3.4.** Levels of MPO protein in the four areas within each of the stool samples were measured by ELISA. Sample 1 (area 1 = 88.93 μg protein/g faeces, area 2 = 98.78 μg protein/g faeces, area 3 = 113.11 μg protein/g faeces, area 4 = 122.01 μg protein/g faeces), sample 2 (area 1 = 76.79 μg protein/g faeces, area 2 = 22.24 μg protein/g faeces, area 3 = 72.2 μg protein/g faeces, area 4 = 113.74 μg protein/g faeces), and sample 3 (area 1 = 0 μg protein/g faeces, area 2 = 0 μg protein/g faeces, area 3 = 6.09 μg protein/g faeces, area 4 = 17.81 μg protein/g faeces) had varying levels of MPO protein.

This result suggests that the homogenisation of all samples prior to extraction would be sensible. This would ensure that the MPO is evenly distributed throughout all the samples.
3.3 Calprotectin and myeloperoxidase levels in patients with inflammatory bowel disease and normal controls

Calprotectin and MPO from 100 samples were measured by ELISA. These results were then grouped into normal controls, patients with CD, and patients with UC. The data was analysed using the Kruskal-Wallis one way analysis of variance on ranks. Figure 3.5 represents levels of calprotectin and MPO protein in these three groups. Calprotectin levels were significantly higher in CD and UC groups compared to the control group (p < 0.001). There was no significant difference between the CD and UC groups. MPO protein levels were significantly higher in the UC group compared to the control group (p < 0.001). However, there was no significant difference between any of the other groups.

![Figure 3.5](image)

**Figure 3.5.** Calprotectin and MPO protein were measured in a total of 100 samples. Of these 100 samples 20 were normal controls, 42 were patients with CD, and 38 were patients with UC. The Kruskal-Wallis analysis of variance on ranks was used to compare levels of calprotectin and MPO protein in the three groups. The lower boundaries of the box plot boxes mark the 25th percentile, the lines within the boxes mark the median. The upper boundaries of the boxes mark the 75th percentile and the whiskers below and above the boxes mark the 10th and 90th percentiles respectively. Dots outside these whiskers represent data values below the 10th percentile or above the 90th percentile. (A) Levels of calprotectin in normal controls (median = 20 μg protein/g faeces, n = 19), CD patients (median = 107.54 μg protein/g faeces, n = 42), and UC patients (median = 181.16 μg protein/g faeces, n = 38). (B) Levels of MPO protein in normal controls (median = 0 μg protein/g faeces, n = 19), CD patients (median = 0 μg protein/g faeces, n = 42), and UC patients (median = 0 μg protein/g faeces, n = 37).
MPO TMB activity was measured using a TMB assay. These results were again grouped into normal controls, patients with CD, and patients with UC. The data was analysed using the Kruskal-Wallis one way analysis of variance on ranks. Figure 3.6 represents MPO TMB activity in the three groups. There was a significant difference between levels of MPO TMB activity in the CD and UC groups (p = 0.015). However, the CD and UC groups did not differ significantly from the control group.

Figure 3.6. MPO TMB activity was measured in a total of 100 samples. Of these 100 samples 20 were normal controls, 42 were patients with CD, and 38 were patients with UC. The Kruskal-Wallis analysis of variance on ranks was used. The figure illustrates MPO TMB activity in normal controls (median = 9.58 μg protein/g faeces, n = 19), CD patients (median = 4.5 μg protein/g faeces, n = 42), and UC patients (median = 8.18 μg protein/g faeces, n = 38).
3.4 Relationships between levels of calprotectin and myeloperoxidase

Calprotectin and MPO protein were measured by ELISA. The levels of the two biomarkers were then correlated against each other. The levels of calprotectin correlated significantly with levels of MPO protein \( (r = 0.434, p << 0.001, n = 98) \), and there was a strong correlation between levels of MPO protein and MPO activity \( (r = 0.751, p << 0.001, n = 98) \). The slope of the graph is approaching 1, indicating that the majority of the MPO is active. Figure 3.7 illustrates the correlations between calprotectin against MPO protein, and MPO protein against MPO activity with scatter plots.

![Graph A](image1)

![Graph B](image2)

**Figure 3.7.** Calprotectin, MPO protein and MPO activity were measured by ELISA in 100 samples from patients with or without IBD. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and MPO protein concentrations \( (r = 0.434, p << 0.001, n = 98) \). (B) Correlation between MPO protein and MPO activity \( (r = 0.751, p << 0.001, n = 98) \).
Calprotectin and MPO protein were also correlated with MPO TMB activity. Calprotectin levels correlated significantly with MPO TMB activity ($r = 0.476, p << 0.001, n = 99$). Similarly, levels of MPO protein also correlated significantly with MPO TMB activity ($r = 0.350, p < 0.001, n = 98$), however, the correlation between calprotectin and MPO TMB activity was stronger. Figure 3.8 illustrates the correlations between calprotectin against MPO TMB activity and MPO protein against MPO TMB activity with scatter plots.

**Figure 3.8.** Calprotectin, MPO protein and MPO TMB activity were measured in 100 samples from patients with or without IBD. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and MPO TMB activity ($r = 0.476, p << 0.001, n = 99$). (B) Correlation between MPO protein and MPO TMB activity ($r = 0.350, p < 0.001, n = 98$).
The relationships between all the variables were assessed using a Spearman’s correlation. Table 3.1 summarises the relationships between all these variables with their corresponding correlation coefficients (r), p values, and n values. Although all 100 samples were used in each of the assays some results could not be obtained and were therefore not included in the statistical analysis.

**Table 3.1. Relationships between levels of calprotectin and myeloperoxidase**

<table>
<thead>
<tr>
<th>Correlation</th>
<th>r-value</th>
<th>p-value</th>
<th>n</th>
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<td>Calprotectin vs. MPO protein</td>
<td>0.434</td>
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<td>98</td>
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<tr>
<td>Calprotectin vs. MPO TMB activity</td>
<td>0.476</td>
<td>&lt;&lt; 0.001</td>
<td>99</td>
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<tr>
<td>MPO protein vs. MPO TMB activity</td>
<td>0.350</td>
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<td>98</td>
</tr>
<tr>
<td>MPO protein vs. MPO activity</td>
<td>0.751</td>
<td>&lt;&lt; 0.001</td>
<td>98</td>
</tr>
</tbody>
</table>
3.5 Relationships between levels of calprotectin and myeloperoxidase in patients with Crohn’s disease and Ulcerative colitis

The relationships between levels of calprotectin and MPO were compared in patients with CD and UC. The CD group contained 42 patients, and the UC group contained 38. In patients with CD calprotectin levels correlated significantly with MPO protein levels ($r = 0.684$, $p << 0.001$, $n = 42$). Levels of MPO protein also correlated strongly with MPO activity measured by ELISA ($r = 0.697$, $p << 0.001$, $n = 42$). The correlation coefficient was greater than 0.5 indicating that over half of the MPO protein is active. Figure 3.9 illustrates the correlations between calprotectin against MPO protein, and MPO protein against MPO activity with scatter plots.

Figure 3.9. Calprotectin, MPO protein and MPO activity were measured by ELISA in 42 patients with CD. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and MPO protein ($r = 0.684$, $p << 0.001$, $n = 42$). (B) Correlation between MPO protein and MPO activity ($r = 0.697$, $p << 0.001$, $n = 42$).
Calprotectin and MPO protein were also correlated with MPO TMB activity measured by TMB assay in patients with CD. Calprotectin correlated significantly with MPO TMB activity \((r = 0.495, p < 0.001, n = 42)\). Similarly, MPO protein also correlated significantly with MPO TMB activity \((r = 0.552, p < 0.001, n = 42)\). Figure 3.10 illustrates the correlations between calprotectin against MPO TMB activity, and MPO protein against MPO TMB activity with scatter plots.

![Figure 3.10](image)

**Figure 3.10.** Calprotectin, MPO protein and MPO TMB activity were measured in 42 samples from patients with CD. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and MPO TMB activity \((r = 0.495, p < 0.001, n = 42)\). (B) Correlation between MPO protein and MPO TMB activity \((r = 0.552, p < 0.001, n = 42)\).
In patients with UC levels of calprotectin correlated strongly with levels of MPO protein \((r = 0.81, p \ll 0.001, n = 37)\). There was a strong correlation between MPO protein and MPO activity \((r = 0.81, p \ll 0.001, n = 37)\). The correlation coefficient was approaching 1 indicating that the majority of the MPO protein is active. Figure 3.11 illustrates correlations between calprotectin against MPO protein, and MPO protein against MPO activity with scatter plots.

**Figure 3.11.** Calprotectin, MPO protein and MPO activity were measured by ELISA in 38 patients with UC. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and MPO protein \((r = 0.81, p \ll 0.001, n = 37)\). (B) Correlation between MPO protein and MPO activity \((r = 0.81, p \ll 0.001, n = 37)\).

Levels of calprotectin correlated significantly with MPO TMB activity \((r = 0.639, p \ll 0.001, n = 38)\) measured by TMB assay. Similarly to patients in the CD group, calprotectin correlated more strongly with MPO protein than MPO TMB activity. MPO protein also correlated significantly with MPO TMB activity \((r = 0.547, p < 0.001, n = 37)\). Figure 3.12 illustrates the correlations between calprotectin against MPO TMB activity, and MPO protein against MPO TMB activity with scatter plots.
Figure 3.12. Calprotectin, MPO protein (measured by ELISA) and MPO TMB activity were measured in 38 samples from patients with UC. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (B) Correlation between calprotectin and MPO TMB activity ($r = 0.639$, $p << 0.001$, $n = 38$). (B) Correlation between MPO protein and MPO TMB activity ($r = 0.547$, $p < 0.001$, $n = 37$).

The relationships between all the variables were assessed using a Spearman’s correlation. Table 3.2 summarises the relationships between all these variables with their corresponding correlation coefficients ($r$), $p$ values, and $n$ values in patients with CD and UC. All 42 CD samples were used in the statistical analysis. However, in the UC patients one result could not be obtained in the MPO ELISA, therefore it was not included in the statistical analysis.

Table 3.2. Relationships between levels of calprotectin and myeloperoxidase in Crohn’s disease and Ulcerative colitis patients
3.6 Relationships between the biomarkers calprotectin and myeloperoxidase with clinical disease activity indices

The levels of calprotectin, MPO protein, and MPO TMB activity were correlated against clinical disease activity indices. The Harvey-Bradshaw Index (HBI) was used for patients with CD, and the Simple Clinical Colitis Activity Index (SCCAI) was used for patients with UC. In patients with CD there was no significant correlation between calprotectin levels and HBI (r = 0.081, p = 0.609, n = 42), and MPO protein levels and HBI (r = 0.162, p = 0.305, n = 42). Figure 3.13 illustrates the correlations between calprotectin against HBI and MPO protein against HBI with scatter plots.

Figure 3.13. Calprotectin and MPO protein were measured by ELISA in 42 samples from patients with CD. The levels of calprotectin and MPO protein were then correlated with HBI. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and HBI (r = 0.081, p = 0.609, n = 42). (B) Correlation between MPO protein and HBI (r = 0.162, p = 0.305, n = 42).
MPO TMB activity was measured by a TMB assay and also correlated with HBI. Similarly to calprotectin and MPO protein there was no significant correlation between MPO TMB activity and HBI ($r = 0.177, p = 0.260, n = 42$). Figure 3.14 illustrates the correlation between MPO TMB activity and HBI with a scatter plot.

**Figure 3.14.** MPO TMB activity was measured in 42 samples from patients with CD. These levels were then correlated with HBI. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. The figure illustrates the correlation between MPO TMB activity and HBI ($r = 0.177, p = 0.260, n = 42$).
In patients with UC there was a significant correlation between levels of calprotectin and SCCAI ($r = 0.458$, $p = 0.004$, $n = 38$), and MPO protein levels and SCCAI ($r = 0.554$, $p < 0.001$, $n = 37$). Figure 3.15 illustrates the correlations between calprotectin against SCCAI and MPO protein against SCCAI with scatter plots.

**Figure 3.15.** Calprotectin and MPO protein concentrations were measured by ELISA in 38 samples from patients with UC. These concentrations were then correlated with SCCAI. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and SCCAI ($r = 0.458$, $p = 0.004$, $n = 38$). (B) Correlation between MPO protein and SCCAI ($r = 0.554$, $p < 0.001$, $n = 37$).
MPO TMB activity was measured by a TMB assay and also correlated with SCCAI. Unlike calprotectin and MPO protein there was no significant correlation between MPO TMB activity and SCCAI ($r = 0.220$, $p = 0.183$, $n = 38$). Figure 3.16 illustrates the correlation between MPO TMB activity and SCCAI with a scatter plot.

![Figure 3.16. MPO TMB activity was measured in 38 samples from patients with UC. These levels were then correlated with SCCAI. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. The figure illustrates the correlation between MPO TMB activity and SCCAI ($r = 0.220$, $p = 0.183$, $n = 38$).](image-url)
The relationships between all the variables were assessed using a Spearman’s correlation. Table 3.3 summarises the relationships between all these variables with their corresponding correlation coefficients (r), p values, and n values in patients with CD and UC. The HBI is the index used for patients with CD, and SCCAI is used for patients with UC. All 42 CD samples were used in the statistical analysis. However, one of the samples measured by ELISA from a patient with UC did not obtain a result; therefore it was not included in the statistical analysis.

**Table 3.3.** Relationships between levels of calprotectin and myeloperoxidase with clinical disease activity indices

<table>
<thead>
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<th>Correlation</th>
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<th>p-value</th>
<th>n</th>
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<td>Calprotectin vs. SCCAI</td>
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<tr>
<td>MPO TMB activity vs. SCCAI</td>
<td>0.220</td>
<td>0.183</td>
<td>38</td>
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</tbody>
</table>
3.7 Relationships between the biomarkers calprotectin and myeloperoxidase with endoscopic scores

The concentrations of calprotectin, MPO protein, and MPO TMB activity were correlated against endoscopic disease severity scores. The Simple Endoscopic Score for Crohn’s disease (SES-CD) patients was used, and the Rachmilewitz score was used for patients with UC. In patients with CD there was a significant correlation between calprotectin levels and SES-CD ($r = 0.487$, $p = 0.001$, $n = 41$), and MPO protein levels and SES-CD ($r = 0.483$, $p = 0.001$, $n = 41$). Figure 3.17 illustrates the correlations between calprotectin against SES-CD, and MPO protein against SES-CD.

![Figure 3.17.](image)

**Figure 3.17.** Calprotectin and MPO protein were measured by ELISA in 42 samples from patients with CD. These levels were then correlated with SES-CD. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and SES-CD ($r = 0.487$, $p = 0.001$, $n = 41$). (B) Correlation between MPO protein and SES-CD ($r = 0.483$, $p = 0.001$, $n = 41$).
MPO TMB activity was measured by a TMB assay and also correlated with SES-CD. Unlike calprotectin and MPO protein there was no significant correlation between MPO TMB activity and SES-CD ($r = 0.303$, $p = 0.054$, $n = 41$). Figure 3.18 demonstrates the correlation between MPO TMB activity and SES-CD with a scatter plot.

Figure 3.18. MPO TMB activity was measured in 42 samples from patients with CD. These levels were then correlated with SES-CD. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. The figure illustrates the correlation between MPO TMB activity and SES-CD ($r = 0.303$, $p = 0.054$, $n = 41$).
In patients with UC there was a significant correlation between calprotectin levels and the Rachmilewitz score ($r = 0.677$, $p << 0.001$, $n = 35$), and MPO protein levels and the Rachmilewitz score ($r = 0.552$, $p < 0.001$, $n = 35$). The relationship between levels of calprotectin and MPO protein against endoscopic scores was stronger in UC patients than CD patients. Figure 3.19 illustrates the correlations between calprotectin against the Rachmilewitz score and MPO protein against the Rachmilewitz score with scatter plots.

Figure 3.19. Calprotectin and MPO protein were measured by ELISA in 38 samples from patients with UC. These levels were then correlated with the Rachmilewitz score. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. (A) Correlation between calprotectin and the Rachmilewitz score ($r = 0.677$, $p << 0.001$, $n = 35$). (B) Correlation between MPO protein and the Rachmilewitz score ($r = 0.552$, $p < 0.001$, $n = 35$).
MPO TMB activity was measured by TMB assay and also correlated with the Rachmilewitz score. Similarly to the results from patients with CD there was no significant correlation between MPO TMB activity and the Rachmilewitz score ($r = 0.258$, $p = 0.134$, $n = 35$). Figure 3.20 illustrates the correlation between MPO TMB activity and the Rachmilewitz score with a scatter plot.

Figure 3.20. MPO TMB activity was measured in 38 samples from patients with UC. These levels were then correlated with the Rachmilewitz score. When a sample was analysed more than once, the mean of the results was used. The Spearman correlation was used to examine the relationship between the variables. The figure illustrates the correlation between MPO TMB activity and the Rachmilewitz score ($r = 0.258$, $p = 0.134$, $n = 35$).

The relationships between all the variables were assessed using a Spearman’s correlation. Table 3.4 summarises the relationships between all these variables with their corresponding correlation coefficients ($r$), $p$ values, and $n$ values in patients with CD and UC. The SES-CD is the index used for patients with CD, and the Rachmilewitz score is used for patients with UC. 41 of the 42 CD samples were used in the statistical analysis, as endoscopic results were not available for one of the patients. In the UC patients 35 of the 38 samples were used in the statistical analysis as one of the results could not be obtained in the assays, and two of the patients did not have readily available endoscopic results.
**Table 3.4.** Relationships between levels of calprotectin and myeloperoxidase with endoscopic scores

<table>
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<th>p-value</th>
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<td>0.001</td>
<td>41</td>
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<tr>
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<td>41</td>
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<tr>
<td>MPO TMB activity vs. SES-CD</td>
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</tr>
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<td>Calprotectin vs. Rachmilewitz score</td>
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<td>MPO protein vs. Rachmilewitz score</td>
<td>0.552</td>
<td>&lt; 0.001</td>
<td>35</td>
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<tr>
<td>MPO TMB activity vs. Rachmilewitz score</td>
<td>0.258</td>
<td>0.134</td>
<td>35</td>
</tr>
</tbody>
</table>
3.8 Relationships between the biomarkers calprotectin and myeloperoxidase with disease severity

The 80 IBD samples were grouped into disease severity groups (inactive, mild, moderate and high) according to their endoscopy scores. The data was analysed using the Kruskal-Wallis one way analysis of variance on ranks. CD and UC patients were analysed separately. Calprotectin levels were significantly higher in patients with high disease severity compared to those with inactive disease (p = 0.009). There was no significant difference between any of the other groups. MPO protein levels were also significantly higher in patients with high disease severity compared to those with inactive disease (p < 0.001). However, there was no significant difference between any of the other groups. Figure 3.21 illustrates calprotectin and MPO protein levels in CD patients with inactive, mild, moderate and high disease severity.

![Graphs showing calprotectin and MPO protein levels](image)

**Figure 3.21.** Calprotectin and MPO protein were measured by ELISA in 42 samples from patients with CD. These levels were then grouped into disease severity according to the SES-CD. The Kruskal-Wallis analysis of variance on ranks was used to compare levels of calprotectin, and MPO protein in the four disease severity groups. (A) Levels of calprotectin concentrations in inactive (median = 70.2 μg protein/g faeces, n = 20), mild (median = 133 μg protein/g faeces, n = 13), moderate (median = 393.5 μg protein/g faeces, n = 5), and high (median = 997.5 μg protein/g faeces, n = 3) disease severity groups. (B) Levels of MPO protein in inactive (median = 0 μg protein/g faeces, n = 20), mild (median = 0 μg protein/g faeces, n = 13), moderate (median = 0 μg protein/g faeces, n = 5), and high (median = 47.97 μg protein/g faeces, n = 3) disease severity groups.
MPO TMB activity was measured by TMB assay and the data was then grouped into disease severity according to SES-CD. Similar to calprotectin and MPO protein, MPO TMB activity was significantly higher in patients with high disease severity compared to those with inactive disease (p = 0.008). However, again there was no significant difference between any of the other groups. Figure 3.22 illustrates MPO TMB activity in CD patients with inactive, mild, moderate and high disease severity.

**Figure 3.22.** MPO TMB activity was measured in 42 samples from patients with CD. The Kruskal-Wallis analysis of variance on ranks was used to compare MPO TMB activity in the four disease severity groups. The figure illustrates MPO TMB activity in inactive (median = 0 μg protein/g faeces, n = 20), mild (median = 6.21 μg protein/g faeces, n = 13), moderate (median = 4.625 μg protein/g faeces, n = 5), and high (median = 50.07 μg protein/g faeces, n = 3) disease severity groups.
In patients with UC levels of calprotectin were significantly higher in patients with high disease severity compared to those with inactive disease ($p = 0.003$). There was no significant difference between any of the other groups. Similarly, levels of MPO protein were significantly higher in patients with high disease severity compared to those with inactive disease ($p = 0.025$). Figure 3.23 illustrates levels of calprotectin and MPO protein in UC patients with inactive, mild, moderate and high disease severity.

![Figure 3.23](image)

**Figure 3.23.** Calprotectin and MPO protein were measured by ELISA in 38 samples from patients with UC. These levels were then grouped into disease severity according to the Rachmilewitz score. The Kruskal-Wallis analysis of variance on ranks was used to compare levels of calprotectin and MPO protein in the four disease severity groups. (A) Levels of calprotectin in inactive (median = 50.38 μg protein/g faeces, $n = 16$), mild (median = 375.5 μg protein/g faeces, $n = 8$), moderate (median = 229.1 μg protein/g faeces, $n = 7$), and high (median = 1139.5 μg protein/g faeces, $n = 4$) disease severity groups. (B) Levels of MPO protein in inactive (median = 0 μg protein/g faeces, $n = 16$), mild (median = 4.34 μg protein/g faeces, $n = 8$), moderate (median = 4.04 μg protein/g faeces, $n = 7$), and high (median = 43.93 μg protein/g faeces, $n = 4$) disease severity groups.
Unlike calprotectin and MPO protein there was no significant difference in MPO TMB activity between any of the disease severity groups (p = 0.501). Figure 3.24 illustrates MPO TMB activity in UC patients with inactive, mild, moderate and high disease severity.

![Myeloperoxidase TMB activity](image)

**Figure 3.24.** MPO TMB activity was measured by TMB assay in 38 samples from patients with UC. These levels were then grouped into disease severity according to the Rachmilewitz score. The Kruskal-Wallis analysis of variance on ranks was used to compare MPO TMB activity in the four disease severity groups. The figure illustrates levels of MPO TMB activity in inactive (median = 5.71 μg protein/g faeces, n = 16), mild (median = 9.03 μg protein/g faeces, n = 8), moderate (median = 6.45 μg protein/g faeces, n = 7), and high (median = 40.67 μg protein/g faeces, n = 4) disease severity groups.

### 3.9 Interspecific variation

The levels of calprotectin, MPO protein and MPO TMB activity were measured again (on a different day) by ELISA and TMB assay in a smaller number of normal controls, CD and UC samples. The initial results and the repeated results were compared using the Mann-Whitney rank sum test. There was no significant difference between the levels of MPO protein measured by ELISA in the initial results compared to the repeated results (p = 0.963, n = 4). A total of 18 samples were repeated, however, only four samples had detectable MPO and could be used in the statistical analysis. There was also no significant difference between the levels of calprotectin measured by ELISA in the initial results compared to the repeated results (p = 0.440, n = 16). A total of 16 samples were repeated and all of these samples had detectable levels of calprotectin and could be used in the statistical analysis.

There was a significant difference between the levels of MPO TMB activity measured by TMB assay in the initial results compared to the repeated results (p = 0.035, n = 14). However, the TMB assay was slightly altered before the second lot of results were obtained (the assay buffer used in the initial
experiment had a lower concentration of BSA than it should of) therefore this could explain the variation in the results, and the experiment would have to be run again under exactly the same conditions to be able to draw any definitive conclusions.
4 Discussion

The aim of this research was to assess if the biomarker MPO measured by ELISA and TMB assay was superior to calprotectin, and could correlate strongly with disease severity in patients with IBD. There was a strong relationship between MPO protein and MPO TMB activity with calprotectin and MPO protein and MPO TMB activity also correlated significantly with disease severity in IBD patients. These results are promising and suggest that there is potential for MPO protein (measured by ELISA) and MPO TMB activity to be used as biomarkers for IBD.

4.1 Myeloperoxidase assay development and optimisation

An essential part of this research was the development and optimisation of the MPO assay. There is limited literature assessing the use of MPO as a biomarker for disease severity in IBD. Therefore, there were few previous extraction techniques available in the literature to follow. MPO is an incredibly sticky protein and as a result its extraction from the stool samples proved to be rather problematic. Preliminary investigations of extraction buffers revealed that PBS + 500 mM NaCl was consistently the most effective extraction buffer for extracting the MPO from the stool samples. However, overall there was no statistically significant difference between the three buffers I tested. Previous research within the Free Radical Research Group indicated that cetrimide may interfere with the ELISA assay; therefore the use of this reagent in the extraction procedure was avoided. When a control sample was spiked with 500 nM MPO and then extracted using the PBS + 500 mM NaCl approximately 24% was recovered. Although this result is low, this extraction buffer again proved to be the most effective, recovering the highest percentage of MPO compared to the other buffers. This low percent recovery means that the results obtained from the MPO ELISA and simple peroxidase assays are a gross underestimation of the total concentration of MPO that is present in clinical samples. However, due to the tight time constraints the extraction procedure could not be optimised any further. Consequently, the PBS + 500 mM NaCl extraction buffer was used to extract the MPO from the 100 clinical samples used in this research as it was consistently the most effective.

The detection of MPO activity by TMB assay was confirmed using the specific MPO inhibitor 2-thioxanthine (79) and including bromide to enhance its activity. 2-thioxanthine successfully inhibited the MPO activity in the three pilot samples I tested. MPO activity was also reduced in all three of the samples in the absence of bromide. MPO and eosinophil peroxidase are the only two enzymes capable of oxidising bromide, therefore, these results confirmed that the activity detected in the samples was predominantly coming from MPO.

MPO distribution appeared to be varied across each part of the faecal sample. However, when the data was normalised and analysis of variance was calculated there was no statistically significant
difference between each of the areas within the stool samples. Despite this result, all clinical samples were homogenised before the extraction procedure to ensure that the MPO was evenly distributed throughout the samples.

4.2 Calprotectin and myeloperoxidase levels in inflammatory bowel disease patients and normal controls

The biomarker calprotectin has been extensively studied, and is routinely used in the diagnosis and follow up of patients with IBD. In this study I found that levels of calprotectin were significantly higher in CD and UC groups compared to normal controls. This same trend has been revealed extensively throughout the literature which helps to further validate my results. MPO protein levels were significantly higher in UC patients compared to normal controls. However, there was no significant difference between MPO protein levels in patients with CD and normal controls. This suggests that MPO protein could potentially be used as an alternative biomarker for discriminating between patients with or without UC. MPO TMB activity measured by TMB assay was capable of discriminating between patients with CD and those with UC, however, the CD and UC groups did not differ significantly from the normal controls.

My research confirmed that MPO protein was not present in any of the normal control patients. This could be explained by five possible scenarios; (1) MPO is only released in the gut when inflammation is present, (2) certain factors trigger the release of MPO in turn causing the inflammation, (3) there is MPO present in normal controls, however, the extraction procedure fails to extract all the MPO that is present, (4) the MPO ELISA assay is not sensitive enough and fails to detect the lower concentrations of MPO that may be present in the samples, or (5) MPO is failing to or only partially binding to the antibody on the ELISA plate.

There is little consensus in the literature with regard to the most appropriate cut-off value for calprotectin. However, in clinical practice a calprotectin concentration of < 50 μg/g is used to indicate that a patient has no active disease (no inflammation present). The majority of the calprotectin levels from the control group fell within this normal range. Having a normal range of 0-50 μg/g suggests that the protein calprotectin is usually present in all individuals, however, concentrations are increased when inflammation is present.

Calprotectin has been shown to correlate with several other biomarkers of inflammation, however, as far as I am aware no alternative biomarker has proven to be more effective in the diagnosis and follow up of patients with IBD. This research demonstrated that there was a significant correlation between calprotectin and MPO protein. Calprotectin and MPO protein also correlated significantly with MPO TMB activity measured by TMB assay. These results are promising and to some extent expected, as
Calprotectin and MPO are both released from neutrophils. There was also a strong correlation between MPO protein and MPO activity indicating that the majority of MPO protein in the gut is active and is potentially contributing to the inflammation present in patients with IBD. When the results were separated into patients with CD and those with UC the relationships between the biomarkers were stronger in UC patients. Previous studies have indicated that biomarkers such as calprotectin appear to reflect disease activity more accurately in UC than CD. This further supports the proposition that MPO could be used as a biomarker of inflammation for patients with UC.

4.3 Correlations with clinical disease activity

The biomarkers calprotectin and MPO were correlated with HBI and SCCAI which are both clinical disease activity indices that are used for patients with CD and UC respectively. There was no significant relationship between calprotectin, MPO protein and MPO TMB activity with HBI. Similarly, there was no significant relationship between MPO TMB activity and SCCAI, however, calprotectin and MPO protein did correlate significantly with SCCAI. Nonetheless, it is important that these results are interpreted with caution due to the numerous problems that exist with these indices. Many variables on the score are subjective, identifying symptoms which are often suffered by patients with irritable bowel syndrome (IBS), as well as those with IBD. A large proportion of the score depends on the patients’ perception of the disease, and this can be influenced by many factors including the euphoric effect of corticosteroids often used in the treatment of CD (45).

4.4 Correlations with endoscopic scores

Numerous studies have shown that calprotectin correlates strongly with endoscopic scores, and can therefore be used to assess the degree of inflammation present in patients with IBD. In this study, I found that there was a significant relationship between calprotectin against SES-CD and calprotectin against the Rachmilewitz score. Similarly MPO protein correlated significantly with SES-CD and the Rachmilewitz score, however, the correlation wasn’t as strong as it was with calprotectin. Unfortunately, there was no significant correlation between MPO TMB activity (measured by simple peroxidase assay) and SES-CD or with the Rachmilewitz score. Colonoscopy with biopsy is currently considered to be the most effective way of determining inflammation location extent and severity. However, this is an invasive procedure and carries risks of complications. The ability of the biomarkers calprotectin and MPO to correlate strongly with endoscopic findings is extremely promising. It confirms that the biomarkers calprotectin and MPO have the potential to be used as an alternative means for assessing inflammation in patients with IBD, or at the very least used alongside endoscopic results to aid in the diagnosis and follow up of patients with IBD.
Similar trends were observed in levels of calprotectin and MPO from patients with CD and UC when they were grouped into disease severity groups (inactive, mild, moderate or high disease severity) based on their endoscopy scores. In patients with CD it was possible to distinguish between inactive and high disease severity using calprotectin and MPO protein levels. However, no differentiation could be made between the inactive, mild and moderate groups. The same was true for patients with UC. In patients with CD MPO TMB activity was significantly higher in patients with high disease severity compared to patients with inactive disease. However, again no discrimination could be made between patients with inactive, mild and moderate disease severity. In patients with UC MPO TMB activity failed to distinguish between varying disease severity. This suggests that calprotectin and MPO protein could be used to discriminate between inactive and high disease severity in patients with IBD (both CD and UC). MPO activity measured by TMB assay could potentially be used to discriminate between inactive and high disease severity in CD patients, its use for discriminating between disease severity in patients with UC is unwarranted at this stage. These findings are promising, however, the results are preliminary and further development and optimisation of both the MPO ELISA and the MPO TMB assay is required to confirm the use of MPO as a biomarker for assessing disease severity in IBD.

4.5 Limitations

During the development and optimisation of the MPO assay there were several problems that arose. The first and potentially most significant was the determination of the most effective extraction buffer. As stated earlier MPO is an incredibly sticky protein and it’s extraction from clinical stool samples proved problematic. The recovery of only 24% MPO from the 500 nM spiked MPO sample suggested the MPO concentrations obtained from the ELISA and TMB assays were a gross underestimation of the total amount of MPO protein present in the stool samples. This could explain why several of the MPO protein values from the 100 clinical samples used gave a concentration of 0 μg/g. The ELISA assay is extremely sensitive and use of certain reagents in the extraction buffers such as cetrimide were avoided due to potential interference with the assay.

Another limitation encountered during the development and optimisation process was the fact that clinical samples from patients with severe IBD were required. Follow up samples were obtained from patients involved in the evaluation of Novel Biomarkers in Inflammatory Bowel Disease project cohort (NBIBD). However, many of the samples coming in at that stage were from normal controls or from patients with IBD that had no active disease at that time. This meant that the number of samples with high disease severity that could be used in the development and optimisation process was very limited and more samples would be needed to validate the results I obtained.
Results from the MPO TMB assay correlated significantly with MPO protein measured by ELISA. However, there were limitations with this assay that need to be considered. Bromide enhances the oxidation of TMB. The two enzymes capable of oxidising TMB are MPO and eosinophil peroxidase. In order to validate the results obtained from this assay a specific inhibitor of eosinophil peroxidase would need to be applied to the TMB reagent before being added to the plate in order to confirm that the peroxidase being detected in the samples was in fact MPO.

Another issue I encountered was the level of sensitivity of the MPO ELISA assay. The assay failed to detect low levels of MPO protein and appeared to only be able to detect MPO in patients with severe disease. This could be due to the limited success of the extraction buffer used as discussed earlier, or could be a result of the MPO protein failing to, or only partially binding to the ELISA plate. A potential way of overcoming this limitation would be to use CM sepharose. CM sepharose is a strong cation (lots of negative charge) that would attract the positively charged MPO protein, a low speed spin and wash steps would then take place. The MPO would therefore be dissociated from the stool sample and bound to the CM sepharose. High salt could then be added to break the ionic interactions. This process would act as a type of purification step in the overall MPO extraction procedure.

The 100 clinical samples used in this research had been collected up to one year prior, aliquoted out into four eppendorf tubes and stored in the -80°C freezer until they were required. Unfortunately, during this process the samples were not homogenised at the time of collection, as a result there may be different concentrations of protein in each of the tubes if the protein of interest was not evenly distributed throughout the sample. To minimise the variation in protein concentrations between different areas within the aliquot of stool used for this research, homogenisation was carried out prior to commencement of the extraction procedure.

Unfortunately, the time constraints prevented the further development of the MPO ELISA and MPO TMB assays. Given more time a more involved extraction procedure could have been developed, potentially extending the vortex and mixing steps to ensure that the MPO is fully dissociated from the stool samples and present in the extraction solution. Re-suspending the pellet after the final spin and repeating the procedure may have also proven to be effective. More extensive investigation is also required into the most effective extraction buffer, with adding a reagent such as CM sepharose to the buffer being a possible option.

The majority of relationships between the biomarkers, disease activity and disease severity were significant, however, the strength of some of these relationships was decreased by a small number of outliers (observations that lie an abnormal distance from the other values). Small sample size could account for the considerable effect that these few outliers had on the overall trends. When the data was split into disease severity groups (based on endoscopy scores) in patients with CD and UC the
numbers in each of the groups became very small. For example in the high disease severity groups in patients with CD and UC n was less than five. This suggests that solid conclusions cannot be drawn from the results obtained, and larger numbers are required to validate my findings. Having an overall sample size of 500 would suggest that even if the data was split into disease severity groups, numbers would likely still be sufficient to draw solid conclusions.

4.6 Future directions and conclusions

There is a growing body of evidence that faecal markers are promising and may be more specific in detecting gut inflammation in patients with IBD. The potential of faecal markers to identify patients with IBD, monitor their treatment outcomes, and to assess their risk of relapse is an appealing prospect. Gastroenterologists would therefore be able to diagnose IBD at a much faster rate by eliminating the wait time of colonoscopy. In addition, they would be able to individualise treatment by prescribing more powerful drugs to patients at risk of relapse, while patients at reduced risk of relapse would avoid these more powerful drugs. The results obtained from this research show that calprotectin and MPO protein correlate strongly with each other. There is also a strong correlation between MPO protein and disease severity, and MPO can successfully distinguish between inactive and high disease severity in CD and UC patients. These results are promising and suggest that MPO may be useful in the diagnosis and follow-up of patients with IBD, however, the MPO ELISA would require further development before it could be used in clinical practice.

Although, the relationships between MPO TMB activity and disease severity in patients with CD and UC were not as significant as they were with calprotectin and MPO protein the results were still very promising. MPO activity measured by a simple peroxidase assay (TMB assay) is extremely fast and cost effective and was shown to correlate strongly with calprotectin and MPO protein measured by ELISA in patients with CD and UC. This means that the use of MPO (measured by simple peroxidase assay) as a biomarker has significant potential to be used in a clinical setting. Patients could provide a sample at commencement of their appointment with a specialised clinician and potentially have a result by the end of their appointment. This would enable patients to be treated at a much faster rate and would eliminate the lag time between sending a sample away to be tested and implementing a treatment plan that best suits the stage of the patients’ disease. If these factors are taken into account it would appear that using MPO measured by a simple peroxidase assay would be a superior biomarker to the current gold standard calprotectin.

However, further development and optimisation of the MPO ELISA and MPO TMB assay is required before their use in a clinical setting can be considered. A more effective extraction buffer, higher sensitivity in both of the assays and larger sample size is essential to validate the results from this research.
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