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Can Laboratory Markers Predict Disease Relapse In Quiescent Crohn's Disease?

by

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Abstract

Maintenance therapy is often used in Crohn's disease to reduce the risk of relapse. An accurate laboratory marker of presymptomatic relapse could allow the selective use of maintenance therapy. The purpose of this study was to examine the predictive value of three potential markers: lactulose/mannitol ratio, postheparin diamine oxidase activity and urinary neopterin excretion. Sixty-one patients with inactive Crohn's disease were recruited and tested three times four months apart and followed monthly for symptomatic relapse. Fourteen subjects (23%) relapsed. Neopterin excretion and diamine oxidase activity were not associated with the risk of relapse. In contrast, an abnormal lactulose/mannitol ratio was associated with a six-fold risk of relapse. Serial testing detected increases in permeability prior to relapse. The test was most sensitive in detecting relapses within 100 days of testing. In conclusion, the lactulose/mannitol test, but not urinary neopterin excretion or plasma postheparin diamine oxidase activity predicts Crohn's disease recurrence.

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List of Abbreviations

DAO	Diamine oxidase
IP	Intestinal permeability
L/M	Lactulose/mannitol ratio
PHDOA	Post-heparin diamine oxidase activity
CDAI	Crohn's Disease Activity Index
PEG	Polyethylene glycol
SD	Standard deviation
^{51}Cr-EDTA	^{51}Cr-labeled ethylenediaminetetraacetic acid
95% CI	95% Confidence Interval

1. Introduction

A. Crohn's Disease

Crohn's disease is a chronic inflammatory condition of the gastrointestinal tract. The disease results in episodes of disease activity, characterized by diarrhea and abdominal pain. Crohn, Ginzberg and Oppenheimer [1] first described the disease in 1932, although isolated cases consistent with the entity were reported throughout the 19th century.

There are marked geographic variations in disease incidence and prevalence. Incidence rates for Crohn's disease increased dramatically from 1955 - 1975, although they have stabilized over the past 15 - 20 years [2]. Incidence rates vary between 0.08 per 100,000 population in Japan to 9.7 per 100,000 population in the Netherlands [3,4]. North American incidence rates range between 3.9 to 4.3 per 100,000 populations [5,6].

Crohn's disease affects predominantly young adults, with a peak age of onset between 15 and 25 years. Women have a slightly greater risk (20 - 30%) of acquiring Crohn's disease compared to men [2]. Crohn's disease is more common among whites than non-whites. Among whites, Jews, especially those of American or European descent, have the greatest risk [7,8].

The cause of Crohn's disease is unknown, although data supports a role for both genetic and environmental factors. A number of potential environmental risk factors have been suspected. These have included diet, cows' milk-feeding and other perinatal events, occupational and social class, and oral contraceptives [9–12]. Strong evidence supporting any of these is lacking. Cigarette smoking has been clearly identified as a risk factor for the disease [13].

A genetic predisposition to Crohn's disease is suggested, not only by the marked variation in prevalence rates among different ethnic groups, but also by evidence from family and twin studies [14–16].

Crohn's disease is a complex clinical entity. It can affect any part of the gastrointestinal tract from mouth to anus and can result in a variety of extraintestinal manifestations. Approximately 35% - 40% of patients will have disease confined to the ileum and cecum, 30% - 40% will have disease confined to the small intestine, and 15% - 25% will have only colonic disease [17]. Pathologically, Crohn's disease is characterized by transmural inflammation, lymphoid aggregates in the submucosa, deep fissures and ulcers, and granulomas.

The predominant symptoms in Crohn's disease are diarrhea, abdominal pain, and weight loss [18]. Other gastrointestinal symptoms can include perianal disease (fistulas, fissures), intestinal obstruction, abscess formation and bleeding. Extraintestinal manifestations include arthritis, skin lesions and kidney stones.

Crohn's disease is usually diagnosed after an individual, who presents with a compatible history, undergoes one or more diagnostic tests. Crohn's disease can be diagnosed by contrast radiography, endoscopy, or at the time of surgery. Though other conditions can mimic Crohn's disease, the diagnosis can generally be made with confidence in most patients. In subjects with purely colonic disease, it may be difficult to distinguish from ulcerative colitis. A final diagnosis of Crohn's disease may only be made after observing the patient over time and identifying features characteristic of Crohn's disease, i.e. perianal disease. The natural history of Crohn's disease is one of periods of

active disease separated by variable periods of quiescence. Even without specific therapy, improvement can occur, with remission rates of 30% after 17 weeks seen in the placebo arms of clinical trials [19].

Patients with active Crohn's disease may be only moderately unwell or may present severely ill or even as an acute surgical emergency. Medical, surgical and nutritional therapy is used in Crohn's disease. The main therapeutic objective is to induce a symptomatic remission by controlling the underlying inflammation. Initial therapy is usually medical with surgical therapy (intestinal resection) reserved for patients whose disease is poorly responsive to medical therapy or who suffer a complication (bowel perforation or obstruction). However, by twenty years from the onset of symptoms, nearly 80% of patients will have undergone an operation for Crohn's disease [20].

There is a growing armamentarium for the medical therapy of Crohn's disease. The cornerstones of treatment include sulfasalazine and newer 5-aminosalicylates, corticosteroids, antibiotics, and immunosuppressives [21,22]. These medications can control the disease when used alone or in combination. However, they do have a number of drawbacks. Corticosteroids can be given either orally, rectally or intravenously and are the mainstay of treatment in moderately or severely active cases. Corticosteroids induce remission in approximately 75% of cases, but their use is limited by a number of acute and chronic side effects and poor patient acceptance [19]. Sulfasalazine and the newer 5-aminosalicylates (mesalamine) are given orally or rectally. They are less effective and have a slower onset of action than corticosteroids, and this limits their use to less severe cases [19]. New preparations of mesalamine have fewer side effects than sulfasalazine or

corticosteroids and are more acceptable to patients. Immunosuppressives, such as azathioprine, and antibiotics are used in selected cases.

2. Literature Review

A. Crohn's Disease Recurrence

Crohn's disease is a chronic, incurable condition. Even following the surgical resection of all gross disease, essentially all patients will eventually relapse [23]. This is in contrast to ulcerative colitis, another chronic, idiopathic inflammatory bowel disease, where resection is curative. Recurrent disease leads to further patient morbidity, and the costs in terms of medications, hospitalizations, and time lost from work are high. It is estimated that the annual medical cost in the United States resulting from Crohn's disease is between 1 and 1.2 billion dollars [24]. This feature of Crohn's disease significantly impacts upon the quality of life of patients and influences the therapy offered to patients [25,26]. Because surgery is not curative, it is used conservatively and often recommended only as a last resort [27,28].

B. Definition of Recurrence

Disease recurrence can be defined in a number of ways: recurrence rates will depend on the definition used. Lennard-Jones and Stadler [29] noted three definitions of recurrence: (1) symptoms, (2) symptoms plus radiological or surgical evidence of recurrence, and (3) surgical recurrence. More recently isolated endoscopic recurrence has become a fourth definition [30].

Surgical recurrence is defined as the need for a second operation after a previous "curative" resection. This definition provides the most conservative estimates of disease recurrence, averaging 15% to 30% by 5 years and 40% to 50% after 15 years [31,32].

Endoscopic recurrence, defined as the presence of visible disease at endoscopy, provides the highest recurrence rates. Endoscopic recurrence rates of 72% to 93% have been reported at one year following ileal resection [30,33,34]. However, the majority of these patients were asymptomatic despite the endoscopic presence of disease [33,34]. Symptomatic recurrence is the presence of symptoms compatible with Crohn's disease recurrence. This definition provides intermediate rates of recurrence.

The problem with using a purely symptom based definition of recurrence is that not all symptoms may be related to recurrent Crohn's disease. Diarrhea and other symptoms may be related to the effects of therapy or may be totally unrelated to the disease. For example, diarrhea is common following resection of the terminal ileum due to bile salt malabsorption causing colonic fluid secretion. Therefore, because of the somewhat non-specific nature of symptoms, many studies have required the presence of endoscopic, radiographic, or surgical evidence of disease to diagnose recurrence. This combined clinical definition is associated with overall rates of postoperative recurrence of 20% at 2 years [35]. However, in clinical practice the decision to institute new therapy to treat active Crohn's disease is frequently made based only on the patient's symptoms without performing further tests.

C. Factors Influencing Risk of Recurrence

Demographic or clinical factors predictive of recurrence have not been clearly identified. Early studies examining potential risk factors have been limited by problems inherent in retrospective designs. Furthermore, a selection bias may have influenced the

findings in many of these studies, as they were performed at major referral centres with sicker, more complex cases.

Patient Characteristics

Age and sex do not influence recurrence rates [26]. The role of oral contraceptives is controversial but appears not to be a risk factor for recurrence [36]. Cigarette smoking increases the risk of recurrence [37–39].

Type of Remission

Crohn's disease may enter remission spontaneously, but induction of remission usually results from medical or surgical therapy. Medically treated patients have a higher one-year recurrence risk than surgical patients.

Initial Disease Site

Though reported results vary, overall combined terminal ileal and large bowel disease appears to have a greater risk of recurrence than isolated large bowel disease [26,32].

Duration of Disease

Sachar et al. [26] reported that preoperative duration of disease was associated with the risk of recurrence, with those with the longer duration of disease having the lower risk of recurrence. This may reflect different sub-groups of disease, an indolent form and a more aggressive form.

Type of Surgery

Patients who undergo intestinal resection with formation of an ileostomy are less likely to recur than those who undergo a resection with primary anastomosis [26,40,41]. Extensive or radical surgery is not associated with a lower risk of recurrence [42].

Duration of Remission

Evidence suggests that the duration of a remission is predictive of the subsequent risk of relapse. Lock, Farmer and Fazio [32] found that the risk of recurrence requiring resection was 3.9 per cent per year for the first eight years following resection, after which the risk declined to about 1.4 per cent per year. However, others have argued that for the population at risk, the chance of postoperative recurrence remains more or less constant each year [41]. Gendre et al. [43] found that in subjects receiving placebo the probability of relapse was higher in those with a shorter duration of remission (< 3 months).

D. Maintenance of Remission

Chronic maintenance therapy is commonly used in ulcerative colitis. Approximately 80% of patients with ulcerative colitis will relapse within one year of entering remission [44,45]. This can be dramatically decreased by the use of prophylactic sulfasalazine or 5-aminosalicylates [46,47].

Placebo-controlled, randomized trials of maintenance therapy for Crohn's disease have not provided as definitive an answer. Early studies suggested no benefit from the use of sulfasalazine or corticosteroids for the maintenance of remission [48–50].

Immunosuppressive agents, such azathioprine and 6-mercaptopurine, have been shown to have maintenance-sustaining properties [51]. However, these drugs do have a number of potentially severe side effects [52]. Therefore, though these medications are useful in patients with chronically active or steroid-dependent disease, in those patients whose disease has been recently brought under control through surgical or conventional

medical therapies, most clinicians would be hesitant to start these medications.

With the advent of newer oral 5-aminosalicylate preparations, further studies were performed to evaluate the utility of this product in preventing recurrence. In contrast to sulfasalazine, newer 5-ASA products have release properties more compatible with delivery of the active drug to the most common site of disease—the ileum or more proximal small bowel. Furthermore, these products are generally better tolerated than sulfasalazine and, therefore, allow higher doses to be given. Results of maintenance trials using 5-ASA preparations have been mixed, but overall it appears that the likelihood of clinical relapse is decreased compared with placebo [53,54]. However, not all studies have shown a benefit [55,56] and some studies have suggested that only certain subgroups benefit [43,57]. Studies have shown beneficial results for patients whose remission was induced medically or surgically [58].

A number of factors limit the appeal of maintenance therapy in Crohn's disease compared with ulcerative colitis. First, the one-year relapse rate is much higher in ulcerative colitis than in Crohn's disease (80% vs. 10% - 30%). Second, clinical trials have not shown as consistently good results for maintaining remission in Crohn's disease as in ulcerative colitis: 5-ASA products appear to decrease the risk of relapse in Crohn's disease by about 40% [53]. Third, though generally well tolerated, 5-ASA products do have side effects and long-term experience with the drugs are limited. Fourth, 5-ASA products are expensive, costing approximately $150 for a months supply. The expense and the need to take the medication three to four times per day limits the acceptability to patients and decreases compliance. In summary, compared to ulcerative colitis, fewer

patients with Crohn's disease are at risk for relapse (more patients will take maintenance therapy without any potential benefit), the protective effect of maintenance therapy is less (the number needed to treat to prevent one relapse will be increased), and there are potential risks and definite costs of maintenance therapy.

E. Predicting Relapse in Quiescent Crohn's Disease

Prophylactic therapy would be more attractive to the physician and the patient if treatment could be targeted to those at the greatest risk of relapse. This would result in only those patients with the most to gain taking the medicine. To illustrate this, consider the following hypothetical example. Approximately 30 relapses would be expected in a group of 100 patients with inactive Crohn's disease. If they all received maintenance therapy with a 5-ASA medication, approximately 12 (40%) of these relapses would be prevented at a total cost of about $180,000 ($150/month × 12 months × 100 patients). If instead only the 30 patients at risk for relapse could be identified and placed on maintenance therapy, the same 12 relapses would be prevented but the cost would be reduced to $64,800.

Given the obvious advantages of the ability to predict a relapse, a number of investigators have sought a useful predictive test. A long list of tests have been proposed, but the majority of these have proven unsuccessful in predicting relapse. Non-specific markers of inflammation or intestinal mucosal damage, such as C-reactive protein, serum orosmucoid, platelet count and α_1-antitrypsin, have been suggested as possible predictors of recurrence, but as single tests their predictive value has been poor [59]. Brignola et al.

[60] used discriminant analysis to create a laboratory index for predicting recurrence. This index required the determination of the erythrocyte sedimentation rate, serum acid α_1-acid glycoprotein, and α_2-globulin. The index was reported to have a sensitivity of 71% and a specificity of 100% for predicting relapse in the subsequent 18 months. The index was subsequently modified to include the white blood cell count, C-reactive protein, and α_1-antitrypsin and α_2-globulin was removed [61].

Brignola et al. [62] used the index to detect a group of patients predicted to be at high risk of relapse and randomized them to treatment with methylprednisolone (0.25 mg/kg daily for 6 months) or placebo. During the course of treatment, one of nine steroid treated patient relapsed compared to seven of nine placebo treated patients. This study, therefore, provided the first evidence that not only could subjects at a high risk of recurrence be detected but that institution of therapy could reduce the risk of relapse.

Though the results reported by Brignola suggest that the laboratory index would be clinically useful, it has some drawbacks. First, it requires a needle-poke to obtain the blood sample. Second, all of the components of the index are not readily available at medical laboratories. Third, the combined cost for determining the index would be relatively expensive.

An ideal laboratory marker of recurrence would have a number of characteristics [63]. Clearly it would have to be accurate. Crohn's disease is a chronic disease that, given enough time, will essentially always relapse, and since it would be unreasonable to expect any test to predict recurrence years later, the test would need to be performed serially. Therefore, it would have to be both acceptable to the patient, in terms of safety and

inconvenience, and of relatively low cost. Therefore, a test that could be performed on an outpatient basis without the need for phlebotomy or disrupting the patient's daily activities would be ideal. Recently, three new laboratory tests have been reported that might fulfill these criteria. These are discussed in the following sections.

F. Intestinal Permeability Testing

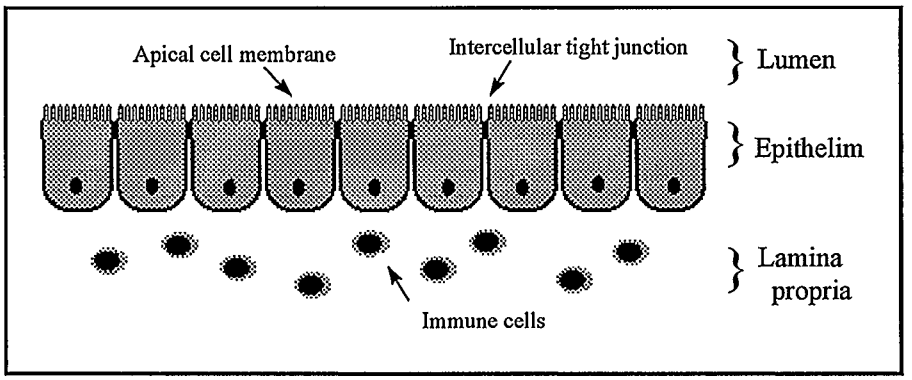
Intestinal Mucosal Barrier

The mucosa of the small and large intestines performs a number of important functions, including the digestion and absorption of nutrients, electrolytes and water. The mucosa also serves an important barrier function, separating the luminal contents from the underlying mucosal interstitial fluid and cells [64,65]. Barriers to the lumen-to-blood transport of solutes includes the unstirred water and mucous layers, the apical and basolateral cell membranes of the epithelial cell, the paracellular junctions, the interstitial matrix, and the capillary and lymphatic endothelia [65]. The barrier function of the epithelium is attributed to closely-apposed, focally-fused cells. The apical lipid bilayer of the cell and the intracellular tight junctions form an effective barrier to water soluble molecules (Figure 1). The barrier function is not complete, however, as passive permeation of solutes via transcellular and paracellular pathways occurs. Molecules with a small radius are able to permeate more rapidly than those with a large radius. The mechanism for this difference is controversial but may be the result of a large population of small "pores" 4 - 7 Å in radius and a smaller population of large "pores" (about 65 Å radius). It is believed that larger molecules must past through the intracellular tight

junctions, whereas smaller molecules may pass through this route but also permeate more rapidly through aqueous pores, which may be transmembrane proteins [66]. Hollander [67] has argued that the permeability characteristics of the intestine could also be consistent with a varying structure and permeability of tight junctions, with 'looser' tight junctions at the villus crypt and 'tighter' junctions at the villus tip.

Figure 1

Intestinal Epithelial Barrier



Permeability Testing

Intestinal permeability can be assessed noninvasively in humans by measuring the urinary excretion of orally administered probes. Probes that are used include polyethylene glycol (PEG), ^{51}Cr-labeled ethylenediaminetetraacetic acid (^{51}Cr-EDTA), disaccharides (lactulose) and monosaccharides (mannitol, rhamnose). Each substance has unique properties that influence its rate of permeation [68,69].

Mannitol has a much greater percent absorption following an oral dose than lactulose. Lactulose is a disaccharide with a larger molecular volume than mannitol. A similar correlation between molecular volume and intestinal permeability is seen for other

test probes, except for PEG which permeates to a greater extent than the others [66]. As discussed above, it has been suggested that these permeability characteristics are consistent with lactulose and other "large" molecules permeating through a paracellular pathway and mannitol permeating through a transcellular route. The large PEG molecule is believed to have a degree of lipid solubility allowing transcellular passage [70]. This interpretation is contrversial, however. Unproven is the presence of transcellular pathways or the transcellular permeation of mannitol. Secondly, investigators have provided conflicting evidence for the lipid solubility of PEG.

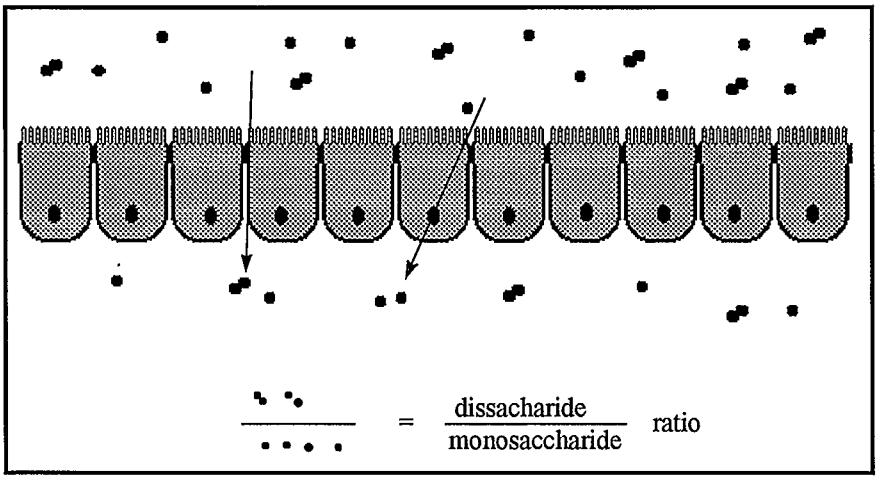
The alternative hypothesis is that passage of these molecules occurs only through a transcellular route [67]. In this theory, a differential structure of intracellular tight junctions results in variable permeability characteristics. A progressive increase in permeability of the tight junctions from the villus tip to crypt is suggested. Since more villus tip is exposed to luminal nutrients, there are a greater number of available small channels. Therefore, fractional excretion of mannitol is normally greater than lactulose.

Though the true routes of marker permeation remains to be proved, the use of these markers to determine intestinal permeability has become widespread. However, when assessing permeability *in vivo* a number of other factors can affect the degree of urinary excretion of the probe [68]. These include the rate of gastric emptying, intestinal transit time, and renal function. Using a single permeability probe, the impact of these other factors on the assessment of intestinal permeability cannot be determined or controlled. To minimize the influence of other factors on the assessment of intestinal permeability, two probes, such as a disaccharide and a monosaccharide, are frequently used. A permeability

index is calculated based on the differential urinary excretion of the probes (Figure 2). Since both probes will be subject to many of the same factors, when the ratio of the excretion of one probe to the other probe is calculated, the influence of other factors is minimized.

Figure 2

Permeability Probe Ratio



The administration of lactulose, a disaccharide, combined with mannitol or rhamnose, monosaccharides, with the calculation of a lactulose/mannitol or lactulose/rhamnose urinary excretion ratio (L/M or L/R) has been widely applied to the study of intestinal permeability in health and disease [66]. Sugar molecules are metabolized by bacteria upon entering the colon, therefore, lactulose/mannitol permeability tests should be a specific measure of small intestinal permeability. Lactulose/mannitol permeability tests are well tolerated and are free of significant side effects.

Intestinal Permeability Testing in Crohn's Disease

Intestinal permeability has been shown to be increased in patients with active Crohn's

disease [71,72]. With successful treatment altered permeability improves [73]. Intestinal permeability tests have been used in many studies of Crohn's disease to assess familial permeability defects in relatives, to determine the effect of therapy and to predict the success of corticosteroid therapy [72–76].

Intestinal Permeability in Predicting Crohn's Disease Relapse

As intestinal permeability is increased in active Crohn's disease and decreases with successful treatment, the predictive value of increased permeability during periods of remission has been queried by investigators. Two abstracts published in 1991 were the first to address this issue. Pironi et al. [77] measured 24 hr urinary excretion of an oral dose of ^{51}Cr-EDTA in 12 Crohn's disease patients following ileocolonic resection and followed the subjects for two years. Three of six subjects with elevated intestinal permeability relapsed within the subsequent 24 months, whereas none of the six with normal intestinal permeability relapsed. Valpiani et al. [78] measured intestinal permeability, using a lactulose/mannitol permeability test, every month for two years. There was a strong correlation between the lactulose/mannitol ratio and the time to relapse.

These early studies have only been published in abstract form. There is limited information provided on the subjects and the follow-up. The study by Valpiani et al. is impressive for the large number of intestinal permeability tests performed. Unfortunately, the data provided in the abstract only looks at relapse over the subsequent 36 days. Predicting a relapse only a few days before it occurs would not be clinically useful, as little could be done at that time to prevent the relapse. It also seems quite unlikely that at the

time of the permeability test a change of symptoms had not already occurred indicating imminent relapse. It would be unusual for subjects to go from an inactive state to a Crohn's Disease Activity Index of greater than 200 (the definition of relapse used in the study) in just a few days. However, these early studies did indicate that further assessment of intestinal permeability in predicting relapse should be done.

Wyatt, Vogelsang, Hübl, Waldhöer, and Lochs [79] assessed the predictive value of a single lactulose/mannitol test. They measured intestinal permeability in 72 patients who had been in remission for at least six months and followed them for one year. Twenty-six of 37 (70%) patients with raised permeability relapsed within one year, compared with 6 of 35 (17%) with normal permeability. Surprisingly, the lactulose/mannitol test was also able to predict relapse in subjects believed to have only colonic disease. Examining the Kaplan-Meier survival curves presented in the paper, suggests that the greatest risk of relapse was seen early (< 3 - 4 months), and that later, even those with normal permeability were at risk for relapse. This suggests that to be a clinically useful predictor of relapse, permeability tests would need to be repeated serially every few months.

G. Plasma Diamine Oxidase Activity

Diamine oxidase is an enzyme located almost exclusively in the villus tip of mammalian enterocytes [80]. Diamine oxidase catabolizes polyamines, although its exact physiologic function is unclear. Normally, plasma levels of diamine oxidase are very low. However, following the injection of intravenous heparin, the enzyme is released into the blood [81]. Luk, Bayless and Baylin [82] have suggested that plasma diamine oxidase

activity may provide a marker of small bowel mucosal integrity. Postheparin plasma diamine oxidase activity is lower than normal in patients with celiac disease, small bowel lymphoma and Crohn's disease [81,83,84] In rats and humans, diamine oxidase is distributed more in the distal small bowel than the proximal small bowel [85,86]: a pattern similar to the distribution of Crohn's disease in humans.

Intestinal diamine oxidase activity correlates with histologic findings. Tissue diamine oxidase activity determined in intestinal resection specimens was significantly less in patients with Crohn's ileitis than in patients with histologically normal small intestine [87]. Crohn's colitis specimens had higher levels than ileitis specimens.

Postheparin plasma diamine oxidase activity is decreased in subjects with Crohn's disease and is inversely correlated to disease activity [85,88]. Induction of remission, medically or surgically, results in an increase in diamine oxidase activity and recurrence is associated with a decrease in activity [89].

Limitations to the clinical use of plasma diamine oxidase activity include the requirement for an intravenous heparin injection and the need for a second blood sample. Heparin facilitates the activity of anti-thrombin III and decreases the blood's clotting ability. The half-life of the anti-coagulant effect of heparin is relatively short, about 90 minutes. However, in a patient population characterized by intestinal ulceration and the propensity for intestinal bleeding, this property is concerning. Initial studies used injections of 15,000 Units of heparin with blood samples drawn over a two hour period. For comparison, a patient receiving heparin to treat a blood clot would typically receive an initial injection of 5,000 - 10,000 units of heparin. Thompson, Burnett, Cormier and

Vaughan [90] showed that a simple regimen, 3000 Units heparin with a single postheparin blood sample drawn 30 minutes later, was adequate to determine diamine oxidase activity. No study, even those using 15,000 Unit boluses or in patients with active disease [84,88], has reported any complication from the heparin injection.

Thompson, Burnett and Vaughan [89] assessed the effect of a number of factors on diamine oxidase activity. Plasma diamine oxidase activity was not related to the extent of small intestinal disease. Diamine oxidase activity in small and large bowel disease was not different. Prior resection of small or large intestine did not significantly alter diamine oxidase activity.

Diamine oxidase activity could potentially predict Crohn's disease recurrence. Evidence supporting this includes the changes in DAO activity seen in subjects in relapse compared to in remission. Furthermore, in one study, the postheparin diamine oxidase activity during periods of active disease was predictive of the risk of relapse in the following year [84].

Given the need for intravenous heparin and blood samples, this test would have to be very accurate to overcome these limitations and be a clinically useful predictive test.

H. Neopterin

Neopterin is a low molecular weight compound derived from quanosine triphosphate (GTP). It is a marker for activated cell-mediated immunity. Enhanced levels of neopterin correlate with stimulation of cell-mediated immune responses [91]. Monocytes and macrophages are the main source of neopterin when activated by interferon-γ produced by

activated T-lymphocytes [92]. Elevation of serum or urine neopterin has been demonstrated in a number of diseases associated with activation of cell-mediated immunity [93,94].

The immune system plays an important role in Crohn's disease [95]. The lamina propria is infiltrated with lymphocytes, macrophages, and other cells of the immune system. The immune cells of the lamina propria are exposed to numerous luminal antigens, which are capable of triggering an immune response.

Prior et al. [96] measured urinary neopterin excretion in 34 patients with Crohn's disease. Neopterin excretion correlated with disease activity. These findings were confirmed in a second study of 76 adults [97]. In one study of 21 pediatric Crohn's disease patients who underwent a total of 135 urinary neopterin determinations over 1 - 43 months of follow-up, neopterin excretion correlated with disease activity [98]. Therefore, it appears that urinary neopterin excretion correlates with disease activity, both between subjects and within subjects over time. Requiring only a timed urine sample, it is easy for the patient to perform.

Serum neopterin or urinary neopterin excretion have not been assessed as possible predictors of recurrence. However, it is known that intestinal inflammation precedes the development of clinical symptoms [34]. Therefore, neopterin excretion may increase prior to symptomatic relapse and may be a useful and simple marker of recurrence.

I. Research Rationale

Crohn's disease commonly recurs following the induction of remission. At present,

maintenance therapy reduces the risk of recurrence but does so by treating a large number of patients to prevent a few relapses. A test that could allow maintenance therapy to be targeted at those with the greatest risk of relapse, while allowing those with a low risk of relapse to avoid the expense, nuisance, and potential risks of medications would be clinically useful.

The lactulose/mannitol test, plasma postheparin diamine oxidase activity and urinary neopterin excretion are three promising markers for identifying presymptomatic Crohn's disease recurrence. A study assessing the predictive value of these markers in patients with medically or surgically induced remission is warranted. Furthermore, a study assessing the three markers at the same time would allow a comparison of the usefulness of the markers alone and in combination.

J. Design Considerations

As Crohn's disease is a chronic disease and patients may relapse even years after entering remission, a predictive test would need to be applied serially over time. But to be useful, the test would have to become positive far enough in advance of clinical relapse to allow therapy to be effective. Therefore, potential markers should be assessed in a longitudinal study with serial measurements.

Prognostic test studies use designs that resemble other observational studies (i.e. cohort studies), but their goals and statistics are different [63]. In assessing a new diagnostic or prognostic test, it is important to study it in much the same way as it would be used in a clinical setting. This means that the subjects studied and outcome predicted

should be representative of those seen in a typical community-based gastroenterology practice.

Outcome

What is the clinically important outcome to predict—recurrence or non-recurrence. This means, in typical clinical practice, would it be more useful to predict with a high degree of certainty which patient is at a high risk for relapse or which patient is at a very low risk for relapse? Given that many studies and two meta-analyses have supported the effectiveness of 5-ASA medications in preventing relapse, it is routine clinical practice for many gastroenterologists to place their patients on these medication as maintenance therapy. Therefore, the potential clinical impact of a prognostic test would be a decrease in the use of maintenance therapy by indicating those patients at a very low risk for relapse and unlikely to benefit from therapy.

Test Characteristics

Tests are commonly evaluated by determining their sensitivity, specificity, positive predictive value and negative predictive value (Table 1) [99,100]. The sensitivity is the proportion of subjects with the disease who have a positive test. The specificity is the proportion of subjects without the disease who have a negative test. The positive predictive value is the probability that a person with a positive result actually has the disease. The negative predictive value of a negative test is the probability that a person with a negative test does not have the disease. Therefore, if the goal is to determine who does not require maintenance therapy, the negative predictive value of a test will be most important. That is the number of people who are falsely identified as not being at risk for

relapse (cell c in Table 1) and who therefore would not be offered maintenance therapy must be small.

Table 1

Characteristics of Diagnostic and Predictive Tests

		True Status		
		Positive	**Negative**	
Test Result	**Positive**	a	b	a + b
	Negative	c	d	c + d
		a + c	b + d	a + b + c + d

Sensitivity = a/(a+c) Specificity = d/(b+d)

Predictive value (positive test result) = a/(a+b)

Predictive value (negative test result) = d/(c+d)

Prevalence = (a+c)/(a+b+c+d)

In contrast to sensitivity and specificity, a test's positive and negative predictive value are influenced by characteristics of the population, especially the prevalence of the disease or outcome [100]. The predictive values found in a study cannot be directly applied to a clinical situation, as the prevalence of the condition in the clinical population may be quite different from that in the study population. As the prevalence of a disease increases, the negative predictive value of a test decreases (Table 2). The negative predictive value will also be decreased if the sensitivity or specificity of a test is decreased.

Table 2

Effect of Changes in Prevalence and Sensitivity on the Predictive Values of a Test

Prevalence	Sensitivity	Specificity	Predictive value positive	Predictive value negative
10%	80%	80%	31%	97%
30%	80%	80%	63%	90%
50%	80%	80%	80%	80%
30%	**60%**	80%	56%	82%

The value of an acceptable negative predictive value would depend on the intended condition being tested for and on the importance placed on a correct diagnosis by the physician and the patient. However, a predictive test to be clinically useful would likely require a negative predictive value of at least 0.80. That is, a negative test would correctly predict at least 80% of the time that a patient would not suffer a recurrence.

3. Objectives

To determine whether intestinal permeability, measured by lactulose/mannitol ratio; plasma post-heparin diamine oxidase activity; or urinary neopterin, alone or in combination, are accurate predictors of disease recurrence in quiescent Crohn's disease.

4. Hypotheses

1. An elevated lactulose/mannitol ratio will indicate a high risk for Crohn's disease recurrence.

2. A low plasma post-heparin diamine oxidase activity will indicate a high risk for Crohn's disease recurrence.

3. An elevated urinary neopterin excretion will indicate a high risk for Crohn's disease recurrence.

4. A combination of altered lactulose/mannitol ratio, plasma post-heparin diamine oxidase activity and/or urinary neopterin excretion will indicate a high risk for Crohn's disease recurrence.

5. Methods

The study was submitted to the Conjoint Medical Research Ethics Board of the University of Calgary Medical Faculty and the Foothill's Hospital Research and Development Committee and received ethical approval prior to initiation.

A. Study Design

Prospective, longitudinal, observational study.

B. Subjects

The target population was patients with small or large bowel Crohn's disease in clinical remission for less than three years with remission induced medically or surgically. The accessible population were those eligible patients living in or near the City of Calgary who were identified between July 1, 1994 and July 1, 1995. Subjects must have fulfilled the following inclusion and exclusion criteria.

Inclusion Criteria

1. Diagnosis of small or large bowel Crohn's disease, based on standard clinical, radiological, endoscopic, pathologic and/or operative findings.
2. Males or females age greater than 18 years.
3. In clinical remission defined as a Crohn's Disease Activity Index score of less than 150 see Section E).
4. Duration of remission at least 30 days but no more than 3 years.
5. Remission induced medically or surgically.

6. If surgical remission, all gross disease removed.
7. On no or stable doses of standard Crohn's disease medications for past thirty days. If on corticosteroids, on no more than the equivalent of 7.5 mg of prednisone.
8. Able to attend the Heritage Medical Research Clinic for initial and follow-up visits.

Exclusion Criteria

1. On regular dose of aspirin or non-steroidal antiinflammatory medications (> 1 dose/week).
2. Recent change in symptoms suggestive of recurrence even if Crohn's Disease Activity Index < 150.
3. Chronic renal insufficiency (serum creatinine > 150 μmol/L).
4. Coagulation disorder (by history and partial thromboplastin time).
5. Diabetes mellitus or glucose intolerance (by history).
6. Ileostomy.
7. Pregnant or lactating.
8. Receiving methotrexate, metronidazole or experimental Crohn's disease therapy.
9. Unable or unwilling to provide signed informed consent.

Sampling and Recruitment and Subject Selection Procedures

Subjects were chosen through nonprobability, convenience sampling. The goal was to obtain a mix of subjects that would be representative of Crohn's disease patients in the community and not only of a tertiary care centre. To achieve this, potential subjects were identified and recruited through several sources.

1. Outpatient gastroenterology clinics at the University of Calgary Medical Clinics.

All patients attending the gastroenterology outpatient clinic at the UCMC are asked to sign a Consent to be Contacted Form if they are interested in participating in clinical research conducted by the members of the Gastroenterology division. During the course of subject recruitment, the patients attending the clinic were reviewed. Patients with Crohn's disease who had signed the consent form were contacted in person at the time of a clinic visit or by phone.

2. Outpatient gastroenterology practices of community gastroenterologists in Calgary.

All gastroenterologists in the city of Calgary were sent an information package about the study and information handouts for interested patients. The physicians were asked to have interested patients contact the study coordinator.

3. University and community-based general and colorectal surgeons.

On a regular basis, the health records department at the Foothill's Hospital and the Calgary General Hospital created a list of all patients who had undergone an intestinal resection for Crohn's disease in the previous two to three months. A list was provided to the surgeons who performed had the procedures. They were asked to contact the patients and request permission from the patient to be contacted about the study.

4. Subject self-identification.

Information about the study was provided publicly through an advertisement in the Crohn's and Colitis Society newsletter, through a talk at the monthly meeting of the Calgary Chapter of the Crohn's and Colitis Society, and through an appearance on a local television station's news program.

5. Inpatients at the Foothill's Provincial Hospital.

Patients admitted to the gastroenterology service at the Foothill's Hospital with an acute exacerbation of Crohn's disease were approached and provided information about the study. The names and addresses of those interested were obtained and they were contacted following discharge and control of their active disease.

All interested patients were first interviewed by phone to explain the nature of the study and assess eligibility criteria. All interested patients who appeared eligible for the study were scheduled for a clinic visit and mailed an information packet that included a description of the study and a copy of the consent form. At the clinic visit, the nature of the study was again reviewed in detail and informed consent was obtained (Appendix 1).

C. Study Protocol

Subjects were seen in the Heritage Medical Research Clinic. At the baseline visit, a questionnaire was completed that covered demographic characteristics, Crohn's disease history, current medications, and tobacco and alcohol use (Appendix 2). Permission was

obtained to access medical records, to confirm the diagnosis and extent of disease, from the subject's physician or from hospitals where care had been received (Appendix 3). At the initial clinic visit, screening bloodwork was obtained to exclude renal insufficiency or coagulopathy. Subjects who remained in remission were seen in the clinic up to two further times, four months apart. Subjects who entered the study late (August 1995) did not undergo a third testing. At each clinic visit, the predictive tests were performed. Subjects were withdrawn from the study if they suffered a relapse (section E). Subjects who were unable to complete further testing for reasons other than disease relapse continued to be followed until the completion of the study.

D. Measurements - Predictor Variables

The three predictive tests assessed were the lactulose/mannitol permeability test, plasma post-heparin diamine oxidase activity, and urinary neopterin excretion.

Permeability Tests

At the time of each clinic visit, subjects performed a lactulose/mannitol permeability test. Subjects were instructed not to take alcohol or non-steroidal anti-inflammatory drugs for at least 24 hours prior to the permeability tests. A test solution was consumed at bedtime following at least a three-hour fast. The test solution contained lactulose 5 g (Technilab, Montreal, Canada), mannitol 2 g (BDH Inc, Toronto, ON, Canada), and sucrose 100 g in 350 mL of water. Subjects voided prior to drinking the test solution and then collected all urine including the first morning void in a pre-weighed container containing 5 mL of 10% thymol in methanol.

Urine samples (10 mL) were deionized by adding 1 g of a 1:1.5 (wt:wt) mixture of Amberlite IR-120 and IRA-400 resin (BDH Chemicals, Toronto ON, Canada). Following centrifugation at 3000 rpm for 10 minutes, the supernatant was filtered through a 40-μm/L Millipore filter (Millipore, Bedford, MA). Samples were separated on a Dionex MA-1 anion exchange column in a Dionex HPLC (Dionex, Oakville, ON, Canada) at room temperature using 500 mmol/L NaOH as the isocratic mobile phase. Peak identification was accomplished with the use of authentic standards and detected using pulsed amperometric electrochemical detection on a gold electrode. Samples were diluted as necessary after addition of cellobiose as an internal standard. Quantitation was performed using known standards at multiple concentrations with linear interpolation between concentrations. All samples were diluted so that concentrations of interest fell within the range of the standards.

The fractional excretion of each probe was determined by calculating the proportion of the ingested probe that was excreted in the urine. The lactulose-mannitol ratio was calculated as a measure of intestinal permeability. The lactulose-mannitol ratio is the ratio of the fractional excretion of lactulose to the fractional excretion of mannitol.

Plasma Post-Heparin Diamine Oxidase Activity

At each clinic visit plasma diamine oxidase activity was determined. A 22 gauge butterfly catheter was inserted into an antecubital vein. Approximately 14 mls of blood was withdrawn into a Vacutainer® tube containing sodium heparin (Beckton Dickinson, Franklin Lakes, NJ). A rapid bolus of injection of 3000 Units of standard, unfractionated porcine heparin sodium (Organon Teknika Inc., Toronto, Canada) was then injected.

Thirty minutes later a second blood sample was drawn. The samples were centrifuged at 3000 rpm for 15 minutes. The supernatant was transferred to clean vials and frozen at minus 20 C.

Diamine oxidase activity was determined using [^{14}C]putrescine as the substrate and measuring the Δ^1-pyrroline formed. DAO activity was measured within six weeks of collection. Preliminary assessment of the stability of samples showed that diamine oxidase activity was retained for six weeks in samples frozen at minus 20 C. 1,4-^{14}C-Putrescine-dihydrocholoride (Sigma, St. Louis, Mo.) was mixed with unlabeled putrescine to give a final concentration of 10 mCi/mmol unlabeled putrescine. This was diluted in Na phosphate buffer (0.1 mmol/l, pH 7.2) to give a final concentration of 0.1 nmol/tube. The reaction mixture consisted of 0.1 μCi 1,4-[^{14}C]putrescine (specific activity, 0.2 nmol/ml), 0.3 ml plasma, and 1.5 ml buffer. Incubation was carried out at 37° C in a shaking waterbath for 1 hour and terminated by boiling for 2 minutes. The mixture was extracted into an equivolume of toluene (2 ml) and shaken vigorously for 20 seconds. One milliliter of organic phase was added to 10 ml of Ecolite (ICN,) and counted in the scintillation counter for 5 min. Samples were assayed concurrently with diamine oxidase standard, 50 - 5U (Sigma, St. Louis, Mo.). One unit of DAO activity equaled 1 μmol of putrescine metabolized per hour at 37° C and pH 7.2.

The post-heparin diamine oxidase activity, in units, was calculated as the change in activity from the baseline to post-heparin samples.

Urinary Neopterin

From the overnight urine collection for the permeability test two 5 ml aliquots were frozen at minus 20 C. All samples were analyzed within six months of collection as per the recommendations of the manufacturer of the assay.

Urinary neopterin levels were determined using a competitive binding radioimmunoassay (INCSTAR, Stillwater, Minnesota). Fifty microliters of urine (diluted 1:10) with 0 standard) were added to 100 µl or ^{125}I neopterin and 100 µL of neopterin primary antibody. The sample was shaken and then incubated at 37° C for 1 hour. Neopterin precipitating complex (500 µL) was then added, and the sample was again shaken and then incubated for 15 minutes at room temperature. Samples were then centrifuged for 20 minutes at 3000*g*, decanted and counted in a gamma scintillation counter for at least 1 minute.

Percent binding for each unknown sample compared to the maximum binding in a 0 standard was determined. This was compared to known standards to determine the final concentration of neopterin in the unknown sample in ng/mL. Neopterin levels were expressed as a ratio to urinary creatinine (ng/ng).

E. Measurements - Outcome Variable

The primary outcome of interest was recurrence of Crohn's disease. As discussed above, recurrence can be defined in many ways. There is no true "gold standard" for diagnosing recurrence. The choice of how to define recurrence in this study was critical. Since this study was evaluating tests that could be used in clinical practice, an outcome

definition that was relevant to clinical practice was important. Surgical recurrence is the most objective definition, however, it is clinically irrelevant as patients are treated for recurrence prior to the need for an operation. Similarly endoscopic recurrence would not be appropriate for this study, as it is not routinely performed and many patients demonstrating endoscopic disease are asymptomatic [101]. Therefore, a clinical definition of recurrence was most appropriate. As previously discussed, symptoms can result from factors other than disease recurrence and many studies have required the demonstration of disease by endoscopy or radiography to diagnose recurrence. However, in clinical practice patients are frequently treated for recurrence based only on symptoms, and repetitive diagnostic tests do subject the patient to some risk. Furthermore, many recent studies of maintenance therapy for Crohn's disease have not used endoscopy or radiology to confirm disease recurrence but have used a definition based on routinely available clinical information [43,55,56,102].

This study uses a definition of recurrence similar to these recent studies, based on detecting an increase in disease activity with the Crohn's Disease Activity Index. Measuring disease activity in Crohn's disease is a controversial and imprecise exercise. A number of disease activity indexes have been developed [103–105]. These generally consider a number of symptoms, signs and laboratory tests in determining a composite score. Often the various indices do not correlate well with each other [106]. None of these scores are perfect; in fact, studies have been conducted that provide somewhat different results depending on which index is used [107].

The Crohn's Disease Activity Index is a widely recognized scoring system. It was developed in the 1970's for the National Cooperative Crohn's Disease Study, one of the first large scale clinical trials of Crohn's disease therapy [108]. It demonstrates construct validity as shown by comparing the score with physicians' over-all ratings [109]. Initially derived from data on 112 patients, the index was subsequently validated on a larger cohort of 1058 patients [105]. Since it was first used as an outcome variable in the National Cooperative Crohn's Disease Study, it has been used in dozens of subsequent studies, including studies of maintenance therapy.

The index is composed of eight components:

Number of liquid or soft stools in 1 week

Sum of 7 daily abdominal pain ratings

Sum of 7 daily ratings of well being

Number of extraintestinal features

Taking opiate antidiarrheal agent

Abdominal mass

Hematocrit

Body weight percent above/below standard weight.

These eight components are readily obtained on most patients. Patients are asked to complete a diary for one week recording number of stools and rating abdominal pain and general well-being each day. In the clinic, the other components are obtained. A relative weighting factor is multiplied by each component and the sum of the products is the final composite score. Generally, mean scores for patients rated as doing very well are around

70, whereas mean scores for patients rated as doing poorly are around 250 [105]. A score of 150 is commonly used as the cut-off between active and inactive disease [108,110].

The CDAI has shown marked interobserver variation, especially when the scores are hand calculated [111]. Not only can errors in calculation result in variation, but also differences in the degree to which extraintestinal manifestations and abdominal masses are elicited can affect interrater reliability.

For this study, subjects were mailed a diary card and requested to complete it in the week preceding their scheduled visit (Appendix 4). At the time of the clinic visit, the patient was weighed by the clinic nurse and the diary was reviewed. A single gastroenterologist questioned and examined all patients at each visit. The presence of extraintestinal features was determined by history and physical exam. The presence of a mass was determined by physical examination and scored as absent, possible or definite. A blood sample was obtained for the hematocrit. The results for each component of the index were entered into a computer spreadsheet program (Microsoft Excel®, Redmond, Wa.) that automatically calculated the components and the total score.

A standard definition of recurrence using the CDAI would be a worsening of the index by at least 100 points and to a total score of greater than 150 for at least two weeks. Some studies have used an increase of 60 points [55,112]. Therefore, an increase of at least 100 points is a more conservative definition. It was chosen to minimize the risk of incorrectly labeling a patient as suffering a recurrence. As many patients (and physicians) are unwilling to persist with this degree of symptoms for two weeks without therapy, two additional diagnostic criteria were added. These stated that recurrence would be

considered to have occurred if the patient required surgery or prednisone in a dose greater than 7.5 mg/day for symptoms compatible with Crohn's disease recurrence prior to the completion of the Crohn's Disease Activity Index. Surgery and corticosteroids are used only after careful consideration by physicians and only for relatively severe symptoms.

To reiterate subjects were considered to have clinical recurrence if they met the following criteria:

1. Increase in the Crohn's Disease Activity Index of at least 100 points and to greater than 150 for two successive weeks.

or

2. Need for prednisone greater than 7.5 mg/day or surgery for symptoms or complications of Crohn's disease.

F. Measurements - Other Variables

Data on a number of other variables was obtained on each subject. These variables were chosen for several purposes: (1) to provide demographic and disease characteristics, (2) to provide data on potential independent risk factors for relapse, and (3) to assess eligibility for the study, and (4) for use in determining the outcome.

Crohn's Disease Activity Index for Survey Research

For between visit follow-up, an abbreviated form of the Crohn's Disease Activity Index was used [113]. No studies have previously indicated a change in this index that determines recurrence. Prior to the initiation of this study, a pilot project was performed which compared the changes in the standard Crohn's Disease Activity Index to the

modified Crohn's Disease Activity Index calculated with data from patients participating in a clinical trial of maintenance therapy. This exercise indicated that an increase of 50 points in the modified scale would be indicative of a relapse in most patients. Therefore, in this study, if the score on the modified index had increased by 50 points from the value at the previous visit, the subject was asked to repeat the diary for another week and was seen in the clinic to determine the actual CDAI score.

Complete Blood Cell Count

At each clinic visit, a complete blood cell count, which includes the hematocrit, platelet count and white blood cell count, was obtained.

Partial thromboplastin time

At the first clinic visit, the partial thromboplastin time was obtained. This was done to exclude an undiagnosed coagulopathy prior to the administration of heparin.

Urinary Creatinine

From each overnight urine sample, a random creatinine was determined.

Serum Creatinine

At the first clinic visit, a serum creatinine was obtained to exclude renal insufficiency.

Disease Characteristics

The site of disease (ileal, ileocolonic, or colonic), duration of disease, type of remission (medical or surgical) and duration of remission was determined at baseline. Use of maintenance therapy was determined. Concomitant illnesses and medications were determined at each visit.

H. Sample Size

The estimated required sample size was calculated based on assumptions derived from the relapse prediction study of Wyatt et al. [79]. It was estimated that 66% of patients with abnormal intestinal permeability would relapse within the subsequent nine months compared to only 11% with normal permeability. Furthermore, it was estimated that approximately 50% of patients with inactive Crohn's disease would have abnormal intestinal permeability. Therefore, to detect a significant difference in the proportion of subjects relapsing with a power of 90% and an alpha of 0.05, it was estimated that at least 29 subjects would need to be recruited.

Recruitment was begun in July 1994. After accrual of the first 20 subjects it was apparent that a sample size of 29 could be insufficient because the number of subjects with abnormal permeability was only about 25%. Therefore, to insure a sufficient sample size and to improve the precision of the study's results, patient recruitment was continued until August 1995. This allowed sufficient time to obtain at least two measurements and 7 - 8 months of follow-up on all subjects.

I. Statistical Analysis

All statistical tests of significance were two-sided with an alpha of 0.050. Analysis was performed using the statistical software Stata (Stata Corporation, College Station, Tx.).

Initial analysis consisted of analysis of baseline characteristics, predictor variables (lactulose/mannitol ratio, neopterin, diamine oxidase activity) and outcome variable

(survival) through univariate and bivariate descriptive statistics and graphs.

The lactulose/mannitol ratio was assessed as a continuous and a dichotomous variable. Subjects were classified as having abnormal permeability if the lactulose/mannitol ratio was greater than 0.0280. This is the upper reference limit defined in a group of healthy controls using the same permeability solution and protocol [114]. No previous study has provided "normal" ranges for diamine oxidase activity or urinary neopterin excretion in Crohn's disease patients. These were assessed as continuous variables.

The primary outcome was disease relapse. Relapse rates were calculated as number of relapses per person-years of follow-up. Subjects were withdrawn from the study at the time of relapses, and subjects were censored at time of last follow-up if they had not met the criteria for relapse. Subjects were also censored if they became ineligible for the study due to no longer meeting the inclusion and exclusion criteria.

Preliminary assessment of relapse rates stratified by baseline variables including remission type, disease site, use of maintenance therapy, and smoking status was conducted with the calculation of rate differences and 95% confidence intervals as described by Rothman [115].

For the purposes of evaluating the study's hypotheses, relapse was examined in three ways. Relapse at any time during follow-up and relapse within 100 days of initial testing were used to assess the predictive value of a single test. Relapse occurring prior to the next clinic visit was used to assess the predictive value of serial testing. Relapse within 100 days was chosen as a measure of short-term relapse. Since all patients with Crohn's disease will eventually relapse and that it is proposed that any predictive test would have

to be performed serially, shorter-term relapse was of greater interest than relapse occurring at any time during follow-up.

To examine relapse at any time during follow-up, standard survival analysis techniques were used [100]. Each predictor variables was first included as the independent variable in a Cox proportional hazards model, in which time until relapse was the dependent variable. Additional variables, including disease site, use of maintenance treatment and smoking status, were also assessed in univariate survival models. Multivariate models were constructed if the univariate model suggested a possible relationship with survival. Smaller models were tested against a full model using the likelihood ratio test. It was recognized that given the limited sample size and number of relapses the ability to detect significant multivariate relationships would be limited.

To examine the predictive utility of serial testing, a Cox regression model that allowed independent variables that varied over time. The dependent variable was cumulative survival time. The predictor variables at each testing time comprised the independent variables. Again variables were included in univariate and multivariate models.

Kaplan-Meier survival curves were created for subjects with normal or abnormal baseline lactulose/mannitol ratios. Differences between the two survival curves were assessed using the Wilcoxon-Gehan and logrank test. Tests such as the Wilcoxon-Gehan test, which are modified versions of the Wilcoxon's rank sum test, tend to be more sensitive than the logrank test to situations where the ratio of hazards is higher at early survival times than at late ones [116].

The short-term predictive value of the tests was assessed by calculating the proportion of subjects that relapsed within 100 days. For the permeability data, the sensitivity and specificity of an abnormal lactulose/mannitol ratio was calculated and the positive and negative predictive value of the test was calculated for several relapse rates.

6. Results

A. Subjects

Sixty-eight potential subjects presented for initial assessment and baseline testing. Seven were excluded because they did not meet eligibility criteria: active disease (3), wrong diagnosis (1), receiving regular nonsteroidal antiinflammatory medication (1), did not complete baseline studies (1) and refusal to participate (1). Therefore, 61 subjects comprised the study group. Subjects were recruited from July 1994 to August 1995.

Table 3

Subject Characteristics

Age, mean (SD)	36 y (11)
Male/female	21/40
Current tobacco use	16 (26%)
Current alcohol use	28 (46%)

Table 3 shows the characteristics of the subjects. The study sample was composed of 40 (66%) women and 21 (34%) males. The mean age for males and females was 36 years. The mean time since the diagnosis of Crohn's disease was 6.7 years.

The site of disease for two subjects who had previously undergone an ileocolonic resection was unknown but was presumed to be ileocolonic. Therefore, 51% of subjects had ileocolonic disease, 38% ileal disease only and 11% colonic disease only. Forty-seven percent of subjects had undergone a previous resection. Current smokers comprised 26% of the study group. Fifty-four percent did not drink alcohol.

Table 4

Remission Characteristics of Study Sample, by Remission Type

	Medical Remission	Surgical Remission	Overall
Number	23 (38%)	38 (62%)	61 (100%)
Duration of remission, median (range)	149 days (31 - 572)	227 days (41 - 1095)	184 days (31 - 1095)
In remission less than 3 months	6 (26%)	8 (21%)	14 (23%)
Crohn's Disease Activity Index, mean	75	62	69
On maintenance therapy	14 (60%)	8 (21%)	22 (36%)

Table 4 shows the remission characteristics for all subjects and stratified by type of remission. Remission had been induced in 23 subjects (38%) medically and in 38 subjects (62%) surgically. The mean time in remission for all subjects was 274 days, with a range of 31 - 1095 days. Twenty-three percent of subjects had been in remission less than 90 days. On average, the duration of the remission was shorter in those in medical remission than those in surgical remission, although a similar proportion had been in remission for less than 90 days. The mean Crohn's Disease Activity Index score was 69, with a range of 0 - 139.

Thirty-six percent of subjects were currently receiving maintenance therapy. The proportion of subjects on maintenance therapy was greater in the medical remission group compared with the surgical remission group (60% vs. 21%). Eighteen (30%) subjects were currently on an oral 5-aminosalicylate preparation, five (8%) were on less than 7.5 mg/day oral prednisone, and two (3%) were on azathioprine or 6-mercaptopurine. All

subjects on medications had been receiving stable doses for at least thirty days, and no subject had recently been placed on medication for a change or worsening of symptoms.

B. Intestinal Permeability

All subjects underwent baseline permeability tests. Forty-one subjects were tested at the second clinic visit. The reasons for not completing a second permeability test included disease relapse (10), moved (3), pregnancy (1), withdrew (4), nausea with first test (1), and did not complete (1). Twenty-three subjects underwent a third permeability test. The reasons for not completing the final test included relapse (3), moved (1), concurrent illness (1), withdrew (1), refused or did not complete (5), late entry into study (7). The results of the lactulose/mannitol test at each visit are summarized in Table 5.

Table 5

Lactulose/Mannitol Ratios at Each Testing Time

	Baseline	**Second Testing**	**Third Testing**
Number tested	61	41	23
Mean	0.0184	0.0179	0.0184
Standard Deviation	0.0112	0.0106	0.0116
Number greater than 0.0280	11 (18%)	6 (15%)	3 (13%)

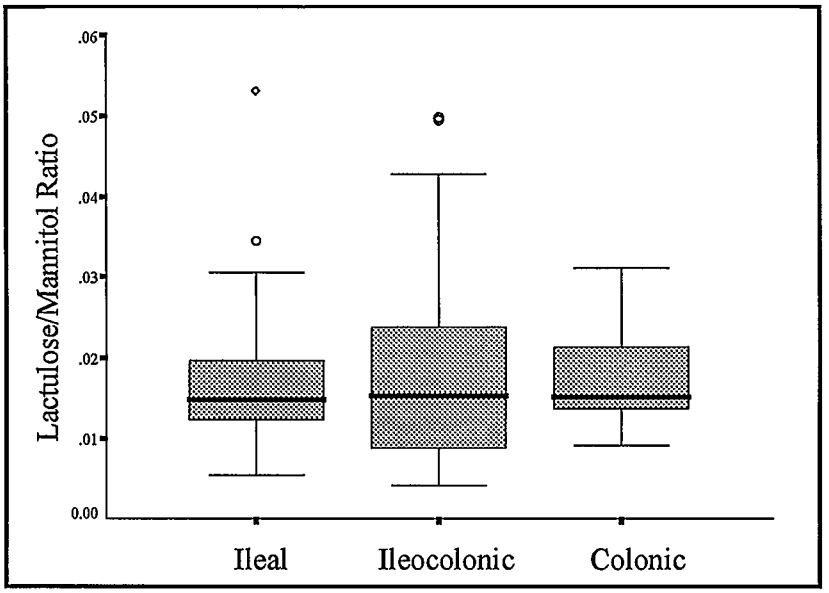
At baseline testing, the mean lactulose/mannitol ratio for the 61 subjects was 0.0184 (95% CI, 0.0155 - 0.0213). For comparison, in a group of healthy controls the mean lactulose/mannitol ratio was 0.0174 [114]. The upper reference limit for this test is 0.0280. Eleven subjects (18%; 95% CI, 9% - 30%) had values greater than 0.0280 and

were defined as having increased intestinal permeability.

The mean lactulose/mannitol ratio was similar in the three disease-site subgroups (Figure 3). The proportion of subjects in each disease site group with abnormal permeability was similar. Four (17%) with ileal disease, 6 (20%) with ileocolonic disease and 1 (14%) with colonic disease had a baseline lactulose/mannitol ratio greater than 0.0280.

Figure 3

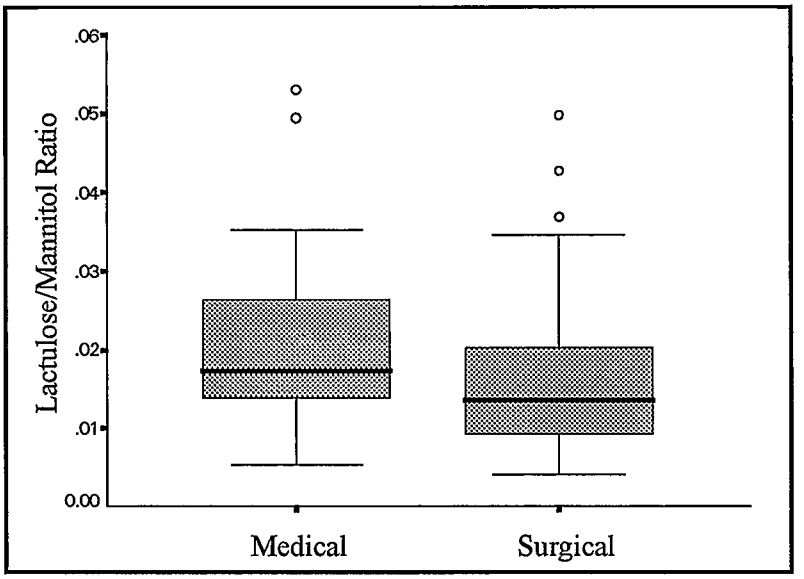
Baseline Lactulose/Mannitol Ratio, by Disease Site



Subjects who entered remission medically had a somewhat higher baseline lactulose/mannitol ratio than those with a surgical remission: 0.021 vs. 0.017 (Figure 4). Furthermore, a greater proportion of subjects in medical remission had abnormal permeability compared to those in surgical remission (26% vs. 13%).

Figure 4

Baseline Lactulose/Mannitol Ratio, by Remission Type



The mean lactulose/mannitol ratio in current smokers and non-smokers was 0.020 and 0.0180, respectively.

Forty-one subjects performed a second permeability test. The mean lactulose/mannitol ratio at second testing was 0.0179. In subjects performing both permeability tests, the mean change from baseline was 0.0005 (range -0.022 - 0.037). At the second visit, six subjects had an abnormally high lactulose/mannitol ratio. Of the six, three had had normal values at baseline testing. At the third testing, 3 of 23 subjects (13%) had high values. Two of the three had had normal values at the two previous testings and one was abnormal at each testing.

C. Post-Heparin Diamine Oxidase Activity

All subjects had post-heparin diamine oxidase activity determined at baseline. Forty-

two underwent a second test and 28 a third test. Table 6 shows the results at the two testing periods.

Table 6

Post-Heparin Diamine Oxidase Activity

	Baseline	Visit 2	Visit 3
Number tested	61	42	28
PHDOA, mean (SD)	15.4 (14.0)	13.1 (12.1)	9.8 (7.2)

For comparison, Thompson, Burnett and Vaughan [89] reported increases in plasma diamine oxidase activity following a similar dose of heparin of 28.5 (SD 30.9) in normal controls. Baseline postheparin diamine oxidase activity were similar for different disease sites and remission types (Table 7).

Table 7

Baseline Post-Heparin Diamine Oxidase Activity, by Disease Site and Remission Type

	PHDOA, mean (SD)
Disease Site	
Ileal	16.7 (15.8)
Ileocolonic	14.9 (13.5)
Colonic	13.4 (9.6)
Remission Type	
Medical	17.7 (16.3)
Surgical	14.0 (12.3)

D. Neopterin

All subjects had determination of urinary neopterin excretion at baseline. Results from 34 subjects from the second testing period are available. Third testing period results are not available. Neopterin excretion is expressed as the ratio of urine neopterin concentration to urine creatinine concentration. The results of urinary neopterin excretion at each visit are summarized in Table 8.

Neopterin excretion was similar when stratified by remission type, disease site and smoking status.

Table 8

Urinary Neopterin Excretion at Each Testing Time

	Baseline	**Second Testing**
Number tested	61	34
Mean, (ng/ng)	206	147
Standard Deviation	241	117
Range	2 - 831	68 - 561

E. Relapse

Subjects were followed to detect the primary outcome of symptomatic disease recurrence. Fourteen subjects (23%) met the criteria for recurrence. This gave an overall risk of relapse of 0.28 per person-year of follow-up. The earliest relapse occurred at day 64 and the latest at day 289. Five (8%) subjects relapsed within 100 days of entering the study. All subjects remaining in remission were followed for at least 101 days (range 101 - 589 days).

A greater proportion of subjects in medical remission relapsed than those in surgical remission (35% vs. 16%). When considered in terms of person-years of follow-up, there was a trend towards improved relapse-free survival in those with a surgical remission, but the confidence intervals associated with the risk difference were wide. (Table 9).

Table 9

Relapse Risk: Incidence Density, by Remission Type

	Medical	**Surgical**
Relapses	8	5
Person-years	17	32
Incidence density	0.47	0.19

Incidence rate difference = 0.28 (95% CI, -0.07 - 0.64)

$P = 0.10$

There was a significant difference in the relapse risk between those receiving and those not receiving maintenance therapy (Table 10). This paradoxical finding, relapse rates higher in those receiving maintenance therapy, likely reflects the use of maintenance therapy in subjects who were correctly identified by their physician as being at an increased risk for relapse, possibly based on the patients' previous history.

Table 10

Relapse Risk: Incidence Density, by Use of Maintenance Therapy

	Maintenance Therapy	No Maintenance Therapy
Relapses	10	4
Person-years	18	32
Incidence density	0.56	0.13

Incidence rate difference = 0.43 (95% CI, 0.06 - 0.79)

$P = 0.009$

As would be expected, there was a trend towards smokers having a higher relapse rate than non-smokers. The relapse rate was 0.5 per person-year for smokers and 0.22 per person-year for non-smokers.

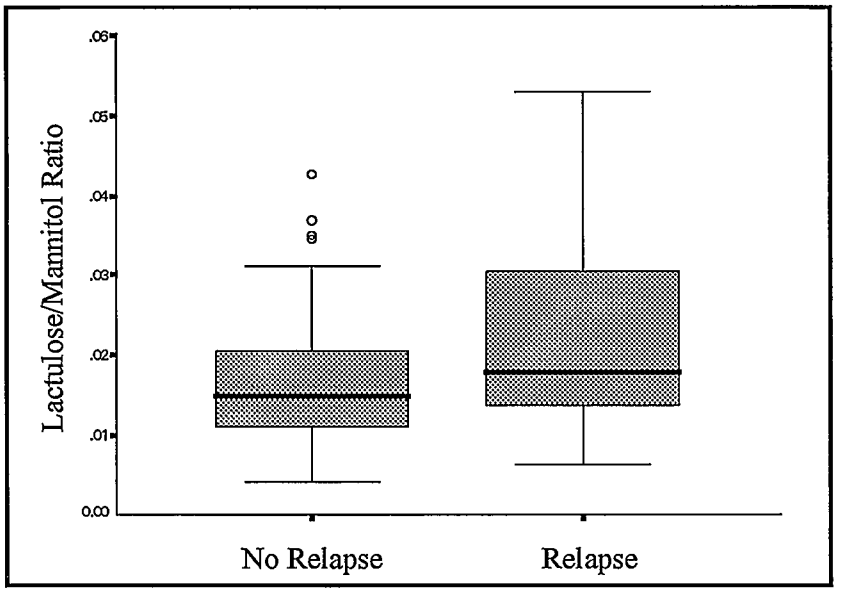
F. Predictive Value of Lactulose/Mannitol Ratio

The first hypothesis to be tested was that subjects with an elevated lactulose/mannitol ratio would have a greater risk of relapse than those with a normal ratio.

The baseline lactulose/mannitol ratio tended to be higher in those subjects who relapsed compared to those who did not (Figure 5). The mean lactulose/mannitol ratio was 0.0168 (0.009) in those who remained in remission and 0.0238 (0.016) in those who relapsed.

Figure 5

Baseline Lactulose/Mannitol Ratio, by Relapse Status



Higher baseline lactulose/mannitol ratios were associated with an increased overall risk of relapse. Table 11 shows the output for a Cox regression model assessing the impact of the baseline lactulose/mannitol ratio on relapse-free survival. This model suggests that the risk of relapse is increased 2.4 times in a patient with a lactulose/mannitol ratio of 0.03 compared to a patient with a ratio of 0.01.

Table 11

Lactulose/Mannitol Ratio and the Prediction of Overall Relapse-Free Survival

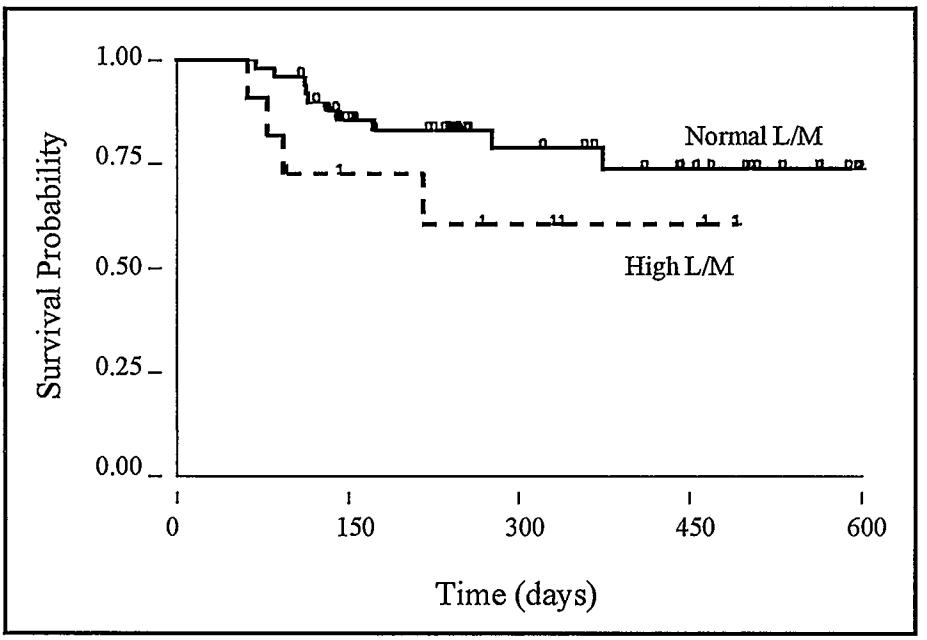
	Coefficient	**95% CI**	**Hazard**	***P***
L/M	0.43	0.04 - 083	1.6	0.031

L/M = Baseline Lactulose/Mannitol × 100

Eleven subjects were classified as having abnormal intestinal permeability (L/M > 0.028) at baseline testing. The relapse incidence density was 0.57 per person-year for those with an elevated lactulose/mannitol ratio and 0.24 per person-year for those with a normal ratio (incidence rate difference 0.33, 95% CI, -0.25 - 0.91). Figure 6 shows the Kaplan-Meier survival curves for relapse stratified by permeability status. Although there was a trend to improved overall relapse-free survival in subjects with a normal baseline permeability test, the two survival curves were not significantly different (logrank, P=0.13). The curves appear most divergent early in follow-up, however, even the Wilcox-Gehan statistic that is more sensitive to early differences in survival did not provide evidence of a difference in survival.

Figure 6

Kaplan-Meier Survival Curves, by Baseline Permeability Status



However, of primary interest was whether serial measurement would improve the predictive value of the lactulose/mannitol test and whether the test was more useful at predicting the short-term risk of relapse.

The value of serial testing was assessed in a Cox proportional hazards model that allowed for time-varying independent variables (Table 12). Using this model, there was evidence that an elevated lactulose/mannitol ratio indicated a greater risk of relapse prior to the next clinic visit (about four months). The risk for a patient with a lactulose/mannitol ratio of 0.03 was 2.7 times the risk for a patient with a ratio of 0.01. When examined in terms of normal or abnormal permeability, those with an elevated lactulose/mannitol ratio had a risk 5.8 times (95% CI, 2.0 - 16.6) greater than those with a normal ratio. Three subjects with normal baseline permeability tests had elevated ratios at the second testing time. Two of the three relapsed subsequently, one at 46 days and the other at 113 days. One of two subjects who developed an elevated ratio at third testing subsequently relapsed.

Table 12

Lactulose/Mannitol Ratio and the Prediction of Relapse-Free Survival, Cox Model Allowing Varying Lactulose/Mannitol Ratio

	Coefficient	**95% CI**	**Hazard**	***P***
L/M	0.46	0.10 - 0.82	1.6	0.011

L/M = Baseline Lactulose/Mannitol Ratio × 100

To assess the short-term predictive value of a single lactulose/mannitol test, relapse within 100 days of the baseline test was examined. Five subjects relapsed within 100 days. The proportion of subjects with an elevated lactulose/mannitol ratio suffering a relapse was significantly greater than those with normal permeability (Table 13). The sensitivity and specificity of the lactulose/mannitol test in predicting relapse within 100 days were 60% and 86%, respectively. Clearly given the small number of relapses the confidence intervals around the point estimates for sensitivity and specificity would be wide.

Table 13

Relapse within 100 days, by Lactulose/Mannitol Ratio

	Normal L/M	**High L/M**
Relapse	2	3
No Relapse	48	8
Risk, (95% CI)	0.04 (0.005 - 0.14)	0.27 (0.06 - 0.60)

Risk ratio = 6.8 (95% CI, 1.29 - 36.1)

P = 0.037

The potential confounding effect of tobacco use on the association between relapse and the lactulose/mannitol ratio was assessed in a Cox regression model. After considering the effect of an elevated lactulose/mannitol ratio on relapse, there was a trend towards current tobacco use being associated with the risk of relapse (Hazard ratio 2.6, $P = 0.08$). Furthermore, the likelihood ratio test did not provide evidence that the inclusion of current tobacco use as a variable improved the fit of the model ($P = 0.1$). However, the sample

size and the limited number of relapses likely provides insufficient statistical power to detect an effect of tobacco use on the risk of relapse.

The inclusion of current smoking in the model did not affect the hazard ratio associated with the lactulose/mannitol ratio. This suggests that the hazard associated with the lactulose/mannitol ratio is not just reflecting the increased risk associated with tobacco use and an affect of smoking on permeability. Therefore, there is no evidence that smoking status is acting as a confounding variable.

G. Predictive Value of Postheparin Diamine Oxidase Activity

The second hypothesis was that low levels of post-heparin plasma diamine oxidase activity would be associated with an increased risk of relapse.

The increase in diamine oxidase activity following heparin for subjects who did or did not relapse is shown in figure 7. The mean increase in diamine oxidase activity in subjects who did not relapse was 16.0 (SD 14.7). For those who relapsed the mean increase was 13.5 (SD 11.4).

Diamine oxidase activity was not associated with the risk of relapse when assessed using Cox regression models to assess the predictive value of a single test or serial tests (Table 14). As diamine oxidase levels vary in the intestine, the predictive value of diamine oxidase was evaluated in a model including disease site and a disease site-diamine oxidase activity interaction term, however, no evidence of an interaction was seen and again no predictive value for diamine oxidase was found. However, the limited number of relapses and patients with purely colonic disease would limit the ability to detect this.

Figure 7

Postheparin Diamine Oxidase Activity, by Relapse Status

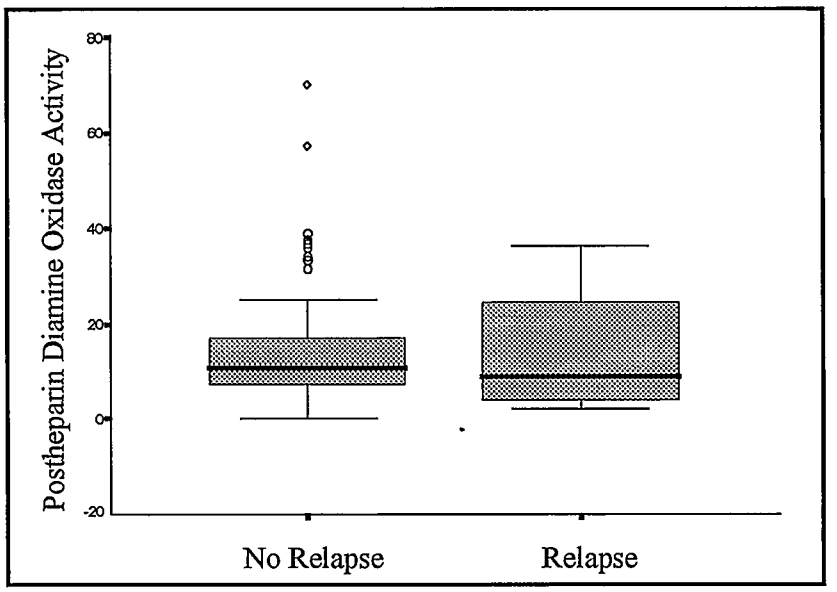


Table 14

Diamine Oxidase Activity and the Prediction of Relapse, Cox Model Allowing Time Varying Diamine Oxidase Activity

	Coefficient (95% CI)	Hazard ratio	
DOA	0.0 (-0.02 - 0.02)	1.0	$P = 0.45$

DOA = Diamine Oxidase Activity × 10

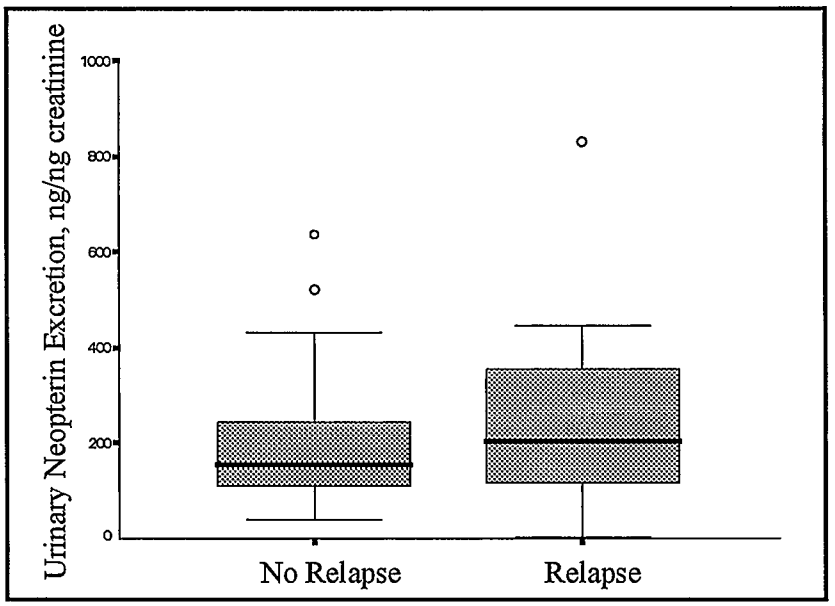
H. Predictive Value of Neopterin Excretion

The third hypothesis to be tested was that elevated urinary neopterin excretion would be associated with an increased risk of relapse.

Urinary neopterin excretion tended to be higher in those who relapsed compared to those who did not, but the variability in the results for both groups was quite large (Figure 8).

Figure 8

Baseline Urinary Neopterin Excretion, by Relapse Status



Neither a single test or serial testing of urinary neopterin excretion predicted relapse when (Table 15).

Table 15

Urinary Neopterin Excretion and the Prediction of Relapse, Cox Model Allowing Time Varying Diamine Oxidase Activity

	Coefficient (95% CI)	Hazard Ratio	
Neo	0.0 (-0.04 - 0.04)	1.0	$P = 0.17$

Neo = Urinary Neopterin Excretion × 10

I. Predictive Value of Combined Tests

The fourth hypothesis to be tested was that a combination of altered lactulose/mannitol ratio, plasma post-heparin diamine oxidase activity or urinary neopterin excretion would be associated with an increased risk of relapse. Given there was already evidence that the an abnormal lactulose/mannitol ratio was predictive of recurrence, this hypothesis was modified to does concurrently determined post-heparin diamine oxidase activity or urinary neopterin excretion improve the prediction of relapse.

This hypothesis was assessed in a Cox proportional hazards model that included a term for normal or abnormal permeability and a continuous variable for the second predictive test. A model including the lactulose/mannitol ratio and either of the other two predictive tests was not associated with a better ability to predict relapse than a model including only the lactulose/mannitol ratio (Table 16).

Table 16

Cox Models Assessing Predictive Value of Diamine Oxidase or Neopterin Excretion in Combination with Lactulose/Mannitol Permeability

A. Cox Model Including the Lactulose/Mannitol Ratio and Diamine Oxidase Activity

	Hazard Ratio (95% CI)	***P***
L/M: high	5.0 (1.7 - 15.0)	0.004
DOA	1.0 (0.9 - 1.0)	0.4

L/M: high = Lactulose/Mannitol Ratio > 0.028

DOA = Diamine Oxidase Activity × 10

B. Cox Model Including the Lactulose/Mannitol Ratio and Neopterin Excretion

	Hazard Ratio (95% CI)	***P***
L/M: high	4.7 (1.5 - 14.2)	0.007
Neo	0.9 (0.9 - 1.0)	0.8

Neo = Urinary Neopterin Excretion × 10

C. Cox Model Including Only Lactulose/Mannitol Ratio

	Hazard Ratio (95% CI)	***P***
L/M: high	5.6 (2.0 - 16.1)	0.001

J. Characteristics of Subjects Whose Relapse was not Predicted by Lactulose/Mannitol Ratio

The only hypothesis that was not rejected was that an elevated lactulose/mannitol ratio would be associated with an increased risk of relapse. Overall the lactulose/mannitol test predicted 7 of the 14 relapses (50%). To determine, in an exploratory fashion, if the relapses of particular patient subgroups were not predicted accurately, the characteristics of those who were and were not predicted by the lactulose/mannitol ratio were assessed. Similar proportions of subjects stratified by remission type, use of maintenance therapy, or smoking status were correctly predicted to be at risk of relapse. When stratified by disease site, some differences were seen. Neither of the subjects with colonic disease who relapsed had elevated lactulose/mannitol ratios. Four of the eight subjects with ileocolonic disease who relapsed had elevated lactulose/mannitol ratios. And finally, three of the four with only ileal disease had an elevated ratio.

The range of time to relapse for those with an elevated lactulose/mannitol ratio was 46 to 133 days, with all but one occurring before 100 days. For those with a normal lactulose/mannitol ratio, the range was 72 to 149 days, with five of seven occurring after 100 days. Therefore, six of eight relapses occurring within 100 days were predicted by the lactulose/mannitol ratio, but only one of six occurring after 100 days was predicted.

7. Discussion

A. Review of Findings

A useful and accurate predictor of imminent Crohn's disease relapse has been elusive. This study assessed three markers with characteristics that indicated they could be clinically useful. Only one, the lactulose-mannitol ratio, was shown to predict recurrence.

Increased intestinal permeability, as demonstrated by an increased lactulose-mannitol ratio, was associated with an increased risk of relapse. For a patient with an abnormal permeability test (>0.028) the risk of relapse was approximately six times greater than for a patient with a normal permeability test. The sensitivity and specificity for a single permeability test predicting recurrence within the subsequent 100 days were 60% and 86%, respectively.

In contrast post-heparin diamine oxidase activity and urinary neopterin excretion did not prove useful in predicting relapse. Diamine oxidase levels were lower in the study subjects than would be expected in a group of subjects without intestinal disease and were similar to values reported in Crohn's disease patients by other investigators [89]. However, a low level of diamine oxidase activity did not predict a relapse. This is in contrast to the study of D'Agostino et al. [84] that suggested that lower levels were associated with a greater likelihood of relapse. They used a different assay for diamine oxidase and, therefore, the values obtained in their patients are not directly comparable to the results of this study. They also used a higher dose of heparin (15,000 Units), although there is no evidence that higher doses provide superior results. However, in that study, diamine oxidase activity was determined at the time of active disease not during remission.

It may have been detecting a subgroup of patients with more aggressive disease, who were, therefore, at a higher risk of relapse than those patients with less aggressive disease.

Urinary neopterin excretion is increased in subjects with active Crohn's disease. In this study neopterin excretion was not associated with an increased risk of relapse. Previous studies of neopterin excretion have used a different method of quantitating neopterin excretion which provides different values for the results. Therefore, there are no studies with which to compare these results. However, this study is similar to other studies of markers of inflammation that have failed to demonstrate a predictive value for a single test [59].

Wyatt et al. [79] previously reported that a single permeability test could predict Crohn's disease relapse. In their study, the sensitivity and specificity of the test in predicting relapse at one year was 81% and 76%, respectively. Their patient population differed from the one in this study as there were more patients in medical remission (70%) and all subjects had been in remission for at least six months. This may explain why they had a higher relapse rate and a greater proportion of subjects with elevated lactulose/mannitol ratios. All of the relapses in Wyatt's study occurred after 90 days of follow-up. In this study, we were able to predict all late recurrences in subjects who performed a second or third permeability test.

B. Threats to Validity and Limitations of the Study

In any study it is important to assess whether the results could have been influenced by any sources of bias. Bias is a systematic error that results in an incorrect estimate of the

association between an exposure and the risk of disease [117]. Bias can be introduced through the methods used to identify and recruit subjects (selection bias), the measurement of information on exposure or outcome (information bias) or through confounding. Confounding occurs when there is a mixing of the effect of the exposure under study on the disease with that of a third factor [118].

Selection bias can be minimized by insuring that the study sample is representative of the larger target population and that the prevalence of the outcome (relapse) in the study sample is not significantly higher than would be expected. A high prevalence of the outcome is frequently seen in subjects recruited from referral centers, as often the patients at these centres are sicker and represent more complicated cases. In this study, subjects were recruited from a variety of sources to insure that the sample was representative of Crohn's disease patients in the community.

Subjects were also not recruited if they believed they were experiencing a change in symptoms suggestive of imminent relapse. Since altered permeability is a characteristic of active disease, it would be anticipated that recruiting such subjects would have overestimated the predictive value of the test.

Measurement bias can be minimized by having clearly defined, objective outcome variables and by blinding the person measuring the outcome to the results of the prediction test. In this study there were clearly defined criteria for diagnosing relapse. All patients were assessed by a single gastroenterologist for the outcome. However, blinding was not complete as some subjects were participating in two studies and the lactulose/mannitol data for the other study was analyzed first. However, there was a limited opportunity for

this knowledge to influence the study's results. To have resulted in a bias, the investigator knowing the permeability results for a portion of the patients would have had to more aggressively elicited extraintestinal manifestations or an abdominal mass in subjects with an elevated lactulose/mannitol ratio, which would have the effect of increasing the likelihood of these patients being classified as relapsed. . However, no subject was classified as a relapse based on these two criteria. The other components of the Crohn's Disease Activity Index and the other criteria for relapse are investigator-independent and could not have been affected by the investigator knowing the results of the permeability test.

It could be argued that the criteria used for classifying relapse could have overestimated the true relapse rate as no objective test of intestinal inflammation such as endoscopy or radiography was performed. This could have introduced a misclassification bias. However, there is no reason to believe that this would have affected one group (high/low permeability) more than another. Therefore, any bias introduced would be expected to effect both groups equally. The effect of a non-differential misclassification bias is to move the estimate of effect towards the null hypothesis [119]. Therefore, rather than falsely indicate that those subjects with an elevated lactulose/mannitol ratio were at greater risk of relapse, it would have reduced the apparent effect.

Confounding can be minimized by measuring variables that may influence the outcome and assessing their influence in the analysis of the results. As discussed, a number of factors are suspected of being associated with the risk of relapse. These include tobacco use, time in remission, type of remission, and use of maintenance therapy. However, to be

a confounder, a factor must be associated with both the exposure and the disease. Except for tobacco use, none of these factors have been associated with changes in any of the predictor tests. The effect of tobacco use on intestinal permeability is controversial, with studies showing variable results [120,121]. In this study, there was no evidence that smoking acted as a confounding factor.

C. Implications of the Study's Findings

Do these results indicate that the lactulose/mannitol test would be a clinically useful test for the prediction of recurrence? To be clinically useful, the lactulose/mannitol test would need to predict recurrence far enough in advance to allow therapy to be initiated that could prevent the relapse. In an earlier study by Valpiani et al. [78] relapse was often predicted only a few days before it occurred. This is unlikely to be sufficient time to allow therapy to be initiated and an effect to be seen. Previous trials of 5-ASA products in active disease suggest that an effect is not seen for approximately one month [122,123], and even corticosteroids often take at least two weeks to show an effect [124]. In this study all relapses predicted by the lactulose/mannitol ratio occurred after 30 days, suggesting that there would be sufficient time to allow treatment to be initiated.

Since Crohn's disease is a chronic disease in which relapse eventually occurs in essentially all patients, a predictive test would have to be applied serially. In this study the lactulose/mannitol test was performed every four months. Five subjects progressed from a normal lactulose/mannitol ratio at baseline testing to an elevated ratio at the second or third testing. Three of these subjects subsequently relapsed. No subject who relapsed after

the second testing time had a normal lactulose/mannitol ratio.

The every four months testing schedule used in this study may not be optimal. Eight subjects relapsed within 100 days of a test. Six of the eight relapses were predicted by an elevated lactulose/mannitol ratio. However, six of the 14 (42%) relapses seen in the study occurred more than 100 days after a test but before the next scheduled test. Five of these six relapses had a normal lactulose/mannitol ratio. This suggests that a shorter interval, possibly three months, between testing would be warranted and may have improved the predictive value of the test in this study.

The sensitivity and specificity of the lactulose/mannitol test in predicting recurrence within 100 days were 0.60 and 0.86, respectively. In this study, the positive predictive value was 0.27 and the negative predictive value was 0.96. The relapse rate was similar to what would be expected in a mixed group of patients with surgically or medically induced remission. Table 16 shows the effect of different relapse probabilities on the expected positive and negative predictive value of the test. In some studies of patients in medical remission relapse rates for placebo-treated patients have been as high as 20% by 100 days [57], whereas for patients in surgical remission the rates have been about 5% at 100 days [58]. In a surgical remission group, the negative predictive value of the test would be expected to be about 98%. However, the pre-test probability of relapse was only 5%, so the additional gain is minimal. In a more mixed population, with a higher expected relapse rate, the negative predictive value would be about 90%. This likely would be clinically useful. Of course, even in the surgical group relapse rates would be higher over a longer period of time, and serial testing would likely be more useful than a single test.

Table 17

Positive and Negative Predictive Value for the Lactulose/Mannitol Test for Different Relapse Probabilities

Prevalence	Positive Predictive Value	Negative Predictive Value
5%	18%	98%
20%	51%	89%

A test that is to be applied serially must be acceptable to the patient. Acceptable not only in terms of a minimal risk of side effects but also in terms of not being inconvenient or unpleasant to perform. Only one subject suffered any kind of adverse event (mild nausea) with the test. Six subjects (about 10%) who completed the first test, refused or did not complete either the second or third permeability test. This suggests that overall the test would be acceptable to patients if used serially. Furthermore, compliance would likely be improved with a modified version of the permeability solution that contains only lactulose and mannitol as many subjects complained about the sweetness of the this test solution that included 100 grams of sucrose. This amount of sucrose is roughly equivalent to putting 25 packets of sugar into a large cup of coffee. The sucrose was included as part of a study that many subjects were also participating in [114].

How should the results of this study be used? Though this study provides evidence that the lactulose/mannitol test can predict recurrence of Crohn's disease in asymptomatic patients, it would be premature to use this test in routine clinical practice. Prediction of relapse is only the first, and possibly easiest, step. The second step is to prevent the

relapse.

Brignola et al. [62] were able to prevent relapses with oral corticosteroids in subjects predicted to be at high risk of relapse by a laboratory index. However, patients dislike using corticosteroids and frequently are loathe to take them even during an acute flare of disease. Also in the study, subjects rapidly relapsed following the withdrawal of the steroids. Therefore, steroids delayed rather than prevented the attack. Previous studies of corticosteroids in patients with inactive disease have suggested that corticosteroids do not prevent recurrences [19]. Studies support a protective effect of 5-aminosalicylates. However, when Brignola et al. [102] tried to duplicate their previous study, this time using mesalamine (5-ASA) in subjects with an abnormal laboratory index, they could not demonstrate a protective effect. This suggests that in subjects predicted to be at high risk of relapse the disease process may have progressed too far for 5-ASA medications to prevent the attack. These patients likely have active inflammatory disease that has not produced symptoms yet.

What could be evaluated in the future is sequential therapy with initial corticosteroids to "induce" remission followed by a 5-aminosalicylate to maintain it. Modigliani et al. [125] have shown that after prednisolone-induced remission, mesalamine facilitates steroid withdrawal and, during the post steroid-weaning year, may reduce the relapse rate. Possibly in these asymptomatic patients a shorter course of steroids would suffice, which would make this protocol more acceptable to patients. The lactulose/mannitol ratio could even be used to guide the duration of therapy, with oral corticosteroids used until the test normalizes and then switching the patient to 5-ASA.

Patients could be evaluated with a front-loaded testing protocol. Permeability testing could initially be performed every two to three months for the first six to twelve months. Those with increased permeability could be placed on therapy, and those who have persistently normal permeability could be switched to a less frequent testing interval.

Of course, the therapy and the testing protocol would need to be evaluated in a clinical trial. In addition, a number of other important questions remain to be answered.

Is the lactulose/mannitol test more accurate in predicting recurrence in certain subgroups of patients? More patients with colonic disease need to be studied to answer this question. Though Wyatt's study [79] suggested that relapse in patients with colonic disease could be predicted only a few patients with purely colonic disease were included. In this study, neither of the relapses in patients with colonic disease were predicted.

Can the lactulose/mannitol test be used in the early preoperative period (first week)? Given the evidence that endoscopic recurrence can occur quickly after surgery [34], many authorities believe that maintenance therapy should be instituted within the first one to two weeks after surgery [126]. No study has assessed the intestinal permeability of Crohn's disease patients immediately after surgery. It is unknown if surgery itself affects permeability. A future study should examine the lactulose/mannitol test in patients soon after surgery. First to assess patient tolerance, and second to determine if and how permeability measurements change in the first few post-operative weeks.

In summary, this study found that the lactulose/mannitol test can predict recurrence of Crohn's disease in asymptomatic patients. In contrast, urinary neopterin excretion and plasma diamine oxidase activity are not useful in predicting recurrence. Furthermore, the

study supported the use of serial testing to decrease the number of false negative tests. The testing interval used appears to have been too long, and testing every two to three months would likely improve the prediction of relapse. In conclusion, the lactulose/mannitol test is a suitable marker of presymptomatic Crohn's disease relapse and could allow the selective use of maintenance therapy in those patients at the highest risk for relapse.

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Appendix 1

CONSENT FORM

Research Project: Laboratory Markers in the Prediction of Crohn's Disease Relapse

Investigators: Drs. R. Hilsden, J. Meddings, L. Sutherland, and J. Wallace

Funding Agency: Departmental Funds

This consent form, a copy of which has been given to you, is only part of the process of informed consent. It should give you the basic idea of what the research project is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included here, you should feel free to ask. Please take the time to read this carefully and to understand any accompanying information.

Crohn's disease frequently recurs after it is brought into remission either by surgical or medical treatment. Recently it has been suggested that the recurrence of symptoms may be prevented by continuous treatment with 5-aminosalicylic acid (Asacol, Pentasa, Salofalk). However, since not all people in remission will relapse, continuous treatment results in the treatment of people who will not benefit from the treatment, but who are still exposed to the small risks and substantial costs of the treatment. Currently there is no standard treatment offered to people in remission.

It may be possible that early disease recurrence can be predicted by laboratory markers in those who are still free of symptoms. If this were true, it would allow selective treatment of only those people who would benefit from the treatment. We plan to evaluate three of these potential markers: intestinal permeability, urinary neopterin, and diamine oxidase activity as to their ability to predict the early recurrence of disease in asymptomatic people with Crohn's disease.

Your participation in this study would involve you drinking a sugar solution and collecting a urine sample three times over a nine month period. Also at the same times a blood test would be performed following the injection of a small dose of heparin, a blood thinner. These tests are explained in more detail below.

The mucosal surface which lines the intestinal tract forms a protective barrier which protects the individual from ingested substances. The "leakiness" of this barrier can be measured by ingesting a mixture of inert, non-absorbable sugars, such as lactulose and mannitol, and then measuring their excretion in the urine. The procedure has no harmful effects. Using such techniques it has been shown that intestinal mucosal "leakiness" or permeability is greater in people with active Crohn's disease and that increased intestinal permeability precedes the return of symptoms.

Neopterin is a product of the bodies inflammatory cells, and diamine oxidase is an enzyme in the intestinal lining. Levels of both are altered in people with active Crohn's disease. Neopterin can be measured in the urine or blood. Diamine oxidase activity can be measured in the blood following an intravenous injection of a small dose of the blood-thinner heparin.

The purpose of our research is to determine whether intestinal permeability, urinary

neopterin, or diamine oxidase activity can predict recurrence of Crohn's disease in asymptomatic patients. Individuals will be assessed initially to confirm disease remission based on a 7-day diary of symptoms and to exclude contraindications to participating in the study. Patients will have initial blood work obtained, including for diamine oxidase activity determinations, following an injection of heparin. That evening the subject will drink the sugar and collect urine the following morning for analysis. These test will be repeated at months four and nine of the study. No other intervention or collection of any kind will be performed.

Limited historical information including name, age, family history, and disease history will be obtained and will be kept confidential. Permission to obtain records of previous operations or investigations may be sought.

Permeability studies have not been associated with any discomfort or short or long term risks. Heparin is an anti-coagulant (blood thinner) and there is a very small, short-lasting (1-3 hours) risk of bleeding. However, in previous similar studies using the same dose of heparin as in this study, no bleeding episodes have been reported. The benefit of this research project will be to determine whether Crohn's disease relapses can be predicted, which would in the future allow selective treatment of only those who are at risk for relapse, rather than all patients in remission. It is our intention to publish the results of our study; however, the patients will be identified only by initials. Participants wishing to, can request a summary of the results of the investigation once it is complete.

Your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to your participation. In no way does this waive your legal rights nor release the investigators, sponsors or involved institutions from their legal and professional responsibilities. You are free to withdraw from the study at any time without jeopardizing your health care. Your continued participation should be as informed as your initial consent so you should feel free to ask for clarification or new information throughout your participation. If you have further questions concerning matters related to this research, please contact Dr. Sutherland at 220-4500, Dr. Meddings at 220-6883, or Dr. Hilsden at 220-6536.

If you have any questions concerning your rights as a possible participant in this research please contact the office of Medical Bioethics, Faculty of Medicine, University of Calgary, at 220-7990.

_____ _____

(Name) (Signature)

_____ _____

(Name of Witness) (Signature)

_____

(Date)

Appendix 2

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

Study ID No.

Patient Initials

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

Study Id No. ☐☐☐ Patient Initials ☐☐☐

Identifying Information

1. Last Name: ☐
   First Name: ☐ Initial: ☐
2. Permanent Address: ☐ (Street)
   ☐ (City, Prov.) ☐ (Postal Code)
3. Telephone Number ☐ (Home) ☐ (Business)
4. Family Physician: ☐
5. Gastroenterologist: ☐

Visit 1

Visit Date ☐ Y ☐ M ☐ D

Demographic Data

1. Birthdate ☐ Y ☐ M ☐ D
2. Sex
   - Male ☐
   - Female ☐
3. Race
   - Caucasian ☐
   - Black ☐
   - Oriental ☐
   - If other, specify, ____________ ☐

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

☐☐☐ Study Id No.

☐☐☐ Patient Initials

Visit 1

Eligibility Criteria

1. Inclusion Criteria

		Yes	No
1.	The patient has signed the informed consent.	☐	☐
2.	The patient is 18 years old or older.	☐	☐
3.	The patient has small or large bowel Crohn's disease demonstrated by **one** of the following. (Must be within last 24 months)	☐	☐
	a. Barium X-ray examination		
	b. Typical gross appearance visualized at surgery		
	c. Visualized by colonoscopy		
	d. Histological appearance at biopsy		
	Date performed ☐☐ Y ☐☐ M ☐☐ D		
4.	The patient has had at least 1 occurrence of acute disease within the last 36 months.	☐	☐
5.	The patient has been in remission for the previous 30 days. (CDAI < 150)	☐	☐
6.	If surgical remission, all gross disease resected.	☐	☐

If the response to any of the above questions is "NO", the patient is **ineligible** to participate in this study.

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

☐☐☐ Study Id No.

☐☐☐ Patient Initials

Visit 1

Eligibility Criteria (cont.)

2. Exclusion Criteria

		Yes	No
1.	Contraindication to heparin. Allergy, bleeding diathesis, recent surgery (1 week), uncontrolled hypertension, proliferative retinopathy, anticoagulant therapy	☐	☐
2.	Short bowel syndrome or patient on TPN.	☐	☐
3.	Active infection or inflammatory process.	☐	☐
4.	Renal insufficiency (creatinine > 150 mmol/L).	☐	☐
5.	Current pregnancy.	☐	☐
6.	Active perianal disease or extraintestinal disease requiring medical therapy.	☐	☐

3. Disallowed Concomitant Medications

Is the patient taking or planning to take the following drug(s)?

1.	Immunosuppressive agents (6-MP, azathioprine, cyclosporine) within the past 6 months.	☐	☐
2.	Chronic (regular use) aspirin or other nonsteroidal anti-inflammatory drugs.	☐	☐

If the response to any of the above questions is "YES", the patient is **ineligible** to participate in this study.

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

Study Id No. | Patient Initials

Visit 1

Medical History

1. History of Crohn's Disease

1. Year the patient was first diagnosed as having Crohn's Disease. 1 9 | |

2. Date of occurrence(s) of acute disease within the last 4 years. (Place in chronological order, beginning with most recent).

Y	M	D

3. Which one(s) of the following was (were) performed to confirm the diagnosis Crohn's Disease?

☐	Barium X-ray examination			
☐	Biopsy			
☐	Appearance visualized at operation			
☐	Colonoscopy			
		Y	M	D

Disease site — Ileitis ☐ Colitis ☐ Ileocolitis ☐ Other small bowel ☐

4. For how many days has the patient been in remission? ☐☐☐ Days

5. Type of remission — Clinical ☐ Bowel resection ☐

6. If surgical remission,	Y	N	Unknown
Was all visible disease removed?	☐	☐	
Were the margins of resection free of disease?	☐	☐	☐

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

Study Id No. ☐☐☐

Patient Initials ☐☐☐

Visit 1

6. Did the patient ever have surgery for Crohn's Disease? Y ☐ N ☐

If yes, date of the most recent surgery

Y ☐☐ M ☐☐ D ☐☐

Name of hospital in which the surgery was performed OR Unknown ☐

_____

_____

May the hospital be contacted to review the patient's records? Y ☐ N ☐

If yes, did the patient sign the hospital's Release of Information Form? ☐ ☐

7. Previous and current drug therapy for Crohn's Disease within the past 18 months

☐ None, Or:

Drug Name	Dose	Regimen	Route	Start Date (Y/M/D)	Date of Last Dose	Response*	Adverse Events**	Continuing Y/N
Codeine								
Lomotil								
Immodium								

* 0 = unchanged or worse; 1 = improved

** 1 = Yes, 2 = No, 3 = Unknown. If yes, specify.

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

☐☐☐ Study Id No.

☐☐☐ Patient Initials

Visit 1

2. General Medical History

1. Current Medical Problems

Other Present Diagnoses	Date of Onset (Y/M/D)

2. Current concomitant drug therapy for other conditions.

☐ None, or:

Drug Name	Dose	Regimen	Route	Start Date (Y/M/D)	Date of Last Dose	Indications	Continuing Y/N

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

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Patient Initials ☐☐☐

Visit 1

3. Tobacco Usage

		Y	N
a) Has the patient ever smoked?		☐	☐
If yes, does the patient smoke currently	Pipe?	☐	☐
	Cigar?	☐	☐
	Cigarettes?	☐	☐
	Number smoked per day	☐☐	
	Period of usage (years)	☐☐	
	Date quit smoking	☐☐ Y	☐☐ M ☐☐ D
	or continuing	☐	
b) Did the patient smoke **before** he/she was diagnosed with Crohn's Disease?		☐	☐
c) Did the patient smoke **at the time** he/she was diagnosed with Crohn's Disease?		☐	☐

4. Alcohol Usage

a) Does the patient currently drink alcohol? ☐ ☐

If yes, how many of each are drunk in an average week?

Bottles of beer? ☐☐

Glasses of wine? ☐☐

Ounces of hard liquor? ☐☐

5. Family History

a) Does the patient have any relatives with Crohn's disease or Ulcerative Colitis?

If yes, specify _____

_____

_____

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

Study Id No. ☐☐☐

Patient Initials ☐☐☐

Visit 1

6. Physical Examination

1. Vital Signs

Height:	☐☐☐.☐ cm.
Weight:	☐☐☐ kg.
Temperature:	☐☐.☐ °C
Seated Blood Pressure:	☐☐☐/☐☐☐ mmHg
Radial Pulse:	☐☐☐ bpm

2. System Review

1. EENT:	Normal ☐	Abnormal ☐
	If abnormal, specify: _____	
2. Cardiopulmonary:	Normal ☐	Abnormal ☐
	If abnormal, specify: _____	
3. Gastrointestinal:	Normal ☐	Abnormal ☐
	If abnormal, specify: _____	
	Mass: No ☐ Possible ☐ Definite ☐	
4. Musculoskeletal:	Normal ☐	Abnormal ☐
	If abnormal, specify: _____	
5. Neuroendocrine:	Normal ☐	Abnormal ☐
	If abnormal, specify: _____	
6. Perianal disease	Yes ☐ No ☐	7. Fistula Yes ☐ No ☐
8. Other, specify:	_____	

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

Study Id No. | Patient Initials

Visit 1

Crohn's Disease Activity Index

		Days 1 2 3 4 5 6 7	Base Score		Weighted Score
1.	Number of liquid or very soft stools			x 2 =	
2.	Abdominal pain (0=none, 1=mild, 2=moderate, 3=severe)			x 5 =	
3.	General well-being (0=gen well, 1=slighthly under par, 2=poor, 3=very poor, 4=terrible)			x 7 =	

4. Extra-intestinal manifestations of Crohn's Disease

	Y	N
arthrits/arthralgia		
iritis/uveitis		
erythema nodosum/pyoderma gangrenosum/apthous stomatitis		
anal fissure, fistula, or abscess		
other fistula		
fever over 37.8° C during past week		

Number of complications ____ x 20 = ____

5. Taking Lomotil/Imodium/opiates for diarrhea (1=yes, 0=No) ____ x 30 = ____

6. Abdominal mass (0=none, 2=questionable, 5=definite) ____ x 10 = ____

7. 47 minus hematocrit _____ (males)
   42 minus hematocrit _____ (females) ____ . x 6 = ____

8. % deviation from standard weight (+ or -) ____ x 1 = ____
   standard weight:
   current weight:

Total CDAI ____

If the CDAI is ≥ 150, the patient is **ineligible** to participate in this study.

Can Laboratory Markers Predict Disease Relapse in Quiescent Crohn's Disease?

Study Id No. ☐☐☐

Patient Initials ☐☐☐

Visit 1

Quality Of Life Assessments

Global Rating Patient

Today my Crohn's disease is:

1 2 3 4 5 6 7

1 = totally inactive
2 = slightly active
3 = somewhat inactive
4 = moderately active
5 = quite active
6 = extremely active
7 = terrible, as bad as it ever gets

Global Rating Physician

Overall, today, this patient's Crohn's disease is:

1 2 3 4 5 6 7

Modified Crohn's Disease Activity Index

		Days 1 2 3 4 5 6 7	Base Score		Weighted Score
1.	Number of liquid or very soft stools	☐☐☐☐☐☐☐	☐☐	x 3 =	☐☐☐
2.	Abdominal pain (0=none, 1=mild, 2=moderate, 3=severe)	☐☐☐☐☐☐☐	☐☐	x 10 =	☐☐☐
3.	General well-being (0=gen well, 1=slighthly under par, 2=poor, 3=very poor, 4=terrible)	☐☐☐☐☐☐☐	☐☐	x 8 =	☐☐☐
				Total CDAI	☐☐☐

Appendix 3

Quiescent Crohn's Disease Study
GI Research Unit
University of Calgary

Date _____

Doctor _____

_____

_____

Please Forward

Pathology Report	☐	_____
Operative Report	☐	_____
Discharge Summary	☐	_____
Colonoscopy Report	☐	_____
Upper Endoscopy Report	☐	_____
Barium Enema Report	☐	_____
Upper GI Series Report	☐	_____

The above-mentioned patient has recently entered the above study. I would appreciate receiving the above information regarding past medical history.

Thank you.

Dr. R. Hilsden
c/o Dr. L. Sutherland
Heritage Medical Research Clinic
3350 Hospital Drive N.W.
Calgary, Alberta
T2N 4N1
Fax: 283-3028

Patient Approval

_____

Appendix 4

Can Laboratory Markers Predict Disease Relapse In Quiescent Crohn's Disease?

Patient Diary

Name:

Since your last review:
1. Any new medications?

2. Any hospitalizations?

3. Any problems related to Crohn's disease?

Instructions
Complete the diary information during seven consecutive days prior to your clinic visit or phone review.

	No. of liquid or very soft stools	Any abdominal pain None = 0 Mild = 1 Moderate = 2 Severe = 3	General Wellbeing Generally well = 0 Slightly below par = 1 Poor = 2 Very poor = 3 Terrible = 4	Loperamide (Imodium) Lomotil or Codeine taken? Yes = 1 No = 0
Day 1				
Day 2				
Day 3				
Day 4				
Day 5				
Day 6				
Day 7				
Totals				

4. Any codeine, Lomotil, or Imodium use in past week?

Date of last diary day _____