The Synergistic Role of Serology, Genetics, and Inflammation in the Diagnosis of INFLAMMATORY BOWEL DISEASE

UC? CD? IBD?
Introduction

Inflammatory bowel disease (IBD) is a chronic and relapsing idiopathic disorder of the gastrointestinal (GI) tract that is characterized by mucosal inflammation and marked by recurrent diarrhea and abdominal pain. While the pathogenesis of IBD is not completely understood, current thinking is that it arises from complex interactions involving the immune system, enteric commensal bacteria, and genetic factors. IBD is divided into two main subtypes of IBD — Crohn’s disease (CD) and ulcerative colitis (UC). While presenting symptoms may be suggestive of one or the other entity, there can be a large overlap of GI symptoms, associated physical findings, and extraintestinal manifestations that are found in both UC and CD that make it difficult to differentiate the two disorders, and thus there is a medical need for adjunctive testing tools to aid clinicians with these diagnostic challenges. This monograph outlines some of the limitations of current IBD diagnostic testing modalities and describes a newly available serologic, genetic, and inflammation biomarker panel that may prove to be beneficial in this process.

Diagnostic Challenges in IBD

Establishing the IBD Diagnosis

At present, there is no “gold standard” test to definitively diagnose IBD. Traditionally, the diagnosis of IBD is based on a combination of data obtained from the patient history and physical examination in association with laboratory, endoscopic, histologic, and radiographic investigations. In adult and pediatric patients, presenting symptoms of IBD are related to both disease location and extent of intestinal inflammation. However, even when symptoms suggest a diagnosis of IBD, they are often too nonspecific to result in a definitive diagnosis, such as in the case of symptom overlap with functional bowel disorders or infectious colitides. This overlap frequently delays an accurate diagnosis of IBD.

Despite the lack of a pathognomonic test for IBD, laboratory values provide useful information in the diagnostic process. Hematologic parameters (increased leukocyte and thrombocyte counts), elevated levels of acute phase reactants (C-reactive protein [CRP] and erythrocyte sedimentation rate), and the presence of fecal markers (leukocytes, lactoferrin, calprotectin) may be associated with active intestinal inflammation, although they do not identify an exact underlying cause. As a result of the limited negative predictive value of laboratory values, the differential diagnosis requires additional testing to rule out alternative disorders including other inflammatory bowel diseases such as those related to infectious agents, medications, radiation, or ischemia; other intestinal disorders (eg, celiac disease, microscopic colitis); or irritable bowel syndrome (IBS).

Establishing the diagnosis of IBD generally relies on the use of invasive testing such as imaging studies and endoscopy. Although multiple imaging techniques are available to aid in the diagnosis of IBD, many are associated with shortcomings from the standpoint of performance. Computed tomographic enterography (CTE) is able to detect clinically occult inflammatory and penetrating disease, but has been reported to have less sensitivity than capsule endoscopy (CE) in detecting ileal inflammation in patients with non-stricturing disease. CTE sensitivity in detecting active colon inflammation has been estimated at 74%. Magnetic resonance enterography (MRE) is associated with suboptimal images in some patients. Like CTE, its ability to detect inflammation is variable, with sensitivity ranging from 55% to 62%. CE has the sensitivity to detect mucosal inflammation of the small bowel, but its use can be complicated by capsule retention due to strictureing disease and may lead to surgical intervention for a retained capsule. In patients with suspected or established CD, the diagnostic yield of CE obtained from case reports, retrospective reviews, and prospective evaluations ranges from 28% to 71%. Ileocolonoscopy is considered the best test for detection of inflammation of the colon and has the added benefit of allowing the collection of biopsy samples for pathologic review. Biopsy results may not prove definitive, however, and the invasive nature of the procedure remains a consideration, especially in the pediatric age group. Ileocolonoscopy is associated with a small risk of perforation (0.03% to 0.15%), as reported in a recent review of nine large clinical series, each of which contained more than 30,000 cases. The need for an adjunctive and comprehensive testing tool is underscored by the challenges related to diagnosing IBD and differentiating CD from UC using a single modality, and with the knowledge that has been gained regarding the known derangements present in IBD (eg, inflammation, immune response to enteric bacteria, genetic factors leading to IBD susceptibility).

Distinguishing Between CD and UC

Differentiating CD from UC is important for its implications in selecting treatment and in the timing and type of surgery that may ultimately be required. However, this differentiation remains a challenge. A recent systematic evaluation by the Swiss IBD Study Group in nearly 1600 patients with diagnoses of CD, UC, or indeterminate colitis (IC) found that diagnostic delay was significantly longer for CD than UC (median 9 vs 4 mo; P <.001). The intervals between first symptoms to physician visit (P =.002), physician visit to diagnosis (P <.001), and first symptoms to IBD diagnosis (P <.001) were all significantly longer for patients with CD than for patients with UC. On multivariate regression analysis, the presence of ileal disease and age less than 40 years at diagnosis were found to be independent risk factors for long diagnostic delay (>24 mo) in patients with CD. In patients with UC, use of nonsteroidal anti-inflammatory drugs was related to a prolonged diagnostic delay (>12 mo) at a trend-level association (P =.093).
Differentiating CD from UC can be difficult in patients whose disease manifestations are exclusively colonic in nature and who lack typical endoscopic or histologic findings, such as in patients with extensive acute, severe colitis. While the main pathologic criterion for the diagnosis of CD is the presence of granulomas, these may not be universally evident in tissue samples obtained either at surgery or at biopsy. A retrospective chart review including 102 patients with CD by Wolfson and colleagues found granulomas in only 45% to 58% of surgically resected specimens examined. An even smaller percentage (as low as 2% to over 20%) is reported to be found in biopsy samples.

**Evaluation of Populations at Risk**

Genetic susceptibility has been implicated in the pathogenesis of IBD. Twin studies carried out in Sweden and Denmark revealed UC concordance rates of 6.3% to 18.2% in monozygotic twins and 3.9% to 4.5% in dizygotic twins. In these same studies, concordance in CD was high for monozygotic twins (58.3%) but not for dizygotic twins (0%). Furthermore, multiple investigations have shown that first-degree relatives of patients with IBD are at an increased risk of developing IBD. In a 2004 review of such studies, Russell and Satsangi reported that the risk of developing CD was highest in siblings of CD probands, where the relative risk was 30 to 42, whereas the overall reported that the risk of developing CD was highest in siblings of CD probands, where the relative risk was 30 to 42, whereas the overall relative risk of developing UC in first-degree relatives of UC probands ranged from 10 to 15.

Results of family and twin studies suggesting a contribution of genetic factors to IBD prompted the search for susceptibility genes in the 1990s. Initial efforts in genetic linkage studies and candidate gene approaches in IBD were slow and gave rise to “susceptibility gene” replication studies. In 2000, efforts to sequence the human genome were followed by the discovery of more than 10 million single nucleotide polymorphisms (SNPs), which are DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered. Many SNPs have no effect on cell function, but scientists believe others could predispose people to disease or influence their response to a drug.

Together, these scientific advances made possible a new type of research effort—the genome-wide association study (GWAS). In a GWAS, the distribution of SNPs is determined in large populations with and without a specific disease. By determining which SNPs co-occur with disease symptoms, researchers can make a statistical estimate of the level of risk associated with each SNP. This cutting-edge research has resulted in the discovery of many susceptibility genes in IBD. Furthermore, genes that confer susceptibility to one or the other constituent diseases—either CD or UC—have also been elucidated, providing a useful tool for diagnostic differentiation.

**Evolution of IBD Testing: PROMETHEUS Laboratories, Inc.**

Biomarkers that differentiate IBD from functional and other bowel disorders, and that can distinguish UC from CD, have the potential to improve the diagnostic process and minimize complications resulting from invasive diagnostic procedures. These biomarkers may be especially advantageous in a pediatric population.

Initially, two serologic markers—ASCA (anti-Saccharomyces cerevisiae antibodies) and pANCA (perinuclear antineutrophil cytoplasmic antibodies)—showed utility in diagnosing IBD and differentiating between UC and CD. ASCA is more prevalent in CD than in UC and healthy controls, and pANCA has more specificity for UC. However, some overlap was noted in that about one fourth of CD patients will express pANCA, and ASCA sensitivity is suboptimal for a definitive diagnosis of CD despite its high specificity. Moreover, pANCA is not highly sensitive for UC because of its overlap in expression in UC-like CD. The low sensitivity of ASCA or pANCA in IBD limits their clinical utility in that a negative test result can rule out neither CD nor UC. Whereas positivity for ASCA and/or pANCA may help assign IC patients a definitive diagnoses of CD or UC, a large percentage of IC patients (85.1%) have been found to be seronegative for both markers.

Efforts to improve the sensitivity of serologic testing in IBD have led to the discovery and validation of new biomarkers and the evolution of diagnostic test panels (Figure 1). The IBD First Step (PROMETHEUS Laboratories, Inc., San Diego, CA) improved the sensitivity of assays for ASCA and pANCA and added a new biomarker, anti-OmpC (antibody to outer membrane porin C), to increase sensitivity for CD. The next generation of this test, IBD Serology 7, was augmented with the recently discovered biomarker anti-CBir1 and incorporated the use of a Smart Diagnostic Algorithm to interpret the overall (versus individual) biomarker interaction results, thus providing a more accurate assessment than previous serologic assays.

In efforts to continue to improve the performance of testing for IBD, as well as the ability to better differentiate between CD and UC, PROMETHEUS Laboratories has now developed the novel and innovative PROMETHEUS IBD sgi Diagnostic test. Designed as an adjunctive test for the workup of suspected IBD and for the differentiation of CD and UC in known IBD, the PROMETHEUS IBD sgi Diagnostic test contains three major classes of biomarkers—serologic, genetic, and inflammatory—that correspond to the complex multidimensional nature of IBD (Figure 2). Genetic markers chosen to be included in the PROMETHEUS IBD sgi Diagnostic test have relevance to various physiologic aspects known to be compromised or impaired in IBD, thus leading to disease susceptibility. Specifically these processes include defective bacterial handling/autophagy, dysregulated signaling pathways, and impaired epithelial barrier function.
**Figure 1.** Evolution of Diagnostic Testing in IBD

<table>
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<th>IBD First Step</th>
<th>IBD Serology 7</th>
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<td>Anti-A4-Fla2</td>
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<td>Anti-OmpC</td>
<td>Anti-Fla-X</td>
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<td>SAA</td>
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ANCA, antineutrophil cytoplasmic antibodies; anti-OmpC, antibodies to outer membrane porin C; ASCA, anti-Saccharomyces cerevisiae antibodies; ATG16L1, autophagy related 16-like 1; CRP, C-reactive protein; DNase, deoxyribonuclease; ECM1, extracellular matrix protein 1; ICAM, intercellular adhesion molecule; IgA, immunoglobulin A; IgG, immunoglobulin G; pANCA, perinuclear antineutrophil cytoplasmic antibodies; SAA, serum amyloid A; STAT, signal transducers and activators of transduction; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

**Figure 2.** IBD occurs under the influence of commensal enteric microflora and mucosal immunity (s) as well as environmental factors in the intestines of genetically susceptible individuals (g) in the presence of inflammatory markers (i).
The text that follows describes the specific serologic, genetic, and inflammatory markers that make up the PROMETHEUS IBD sgi Diagnostic test.

Serologic Markers

Serologic markers are important in CD because their expression represents the host response to translocation of intestinal pathogens into the bloodstream as a result of breakdown of the gut mucosal barrier. Investigations to uncover the underlying cause of the abnormal intestinal inflammatory reaction that characterizes IBD have led to the discovery of antibodies that are present specifically in the blood of patients with CD or UC. Initially, research focused on ASCA, which was associated with CD, and pANCA, which was associated with UC. Given the heterogeneity of IBD, it soon became clear that these biomarkers would not lead to a diagnosis of IBD in all patients who have the disorder, as about 30% are negative for both ASCA and pANCA immune responses. Additionally, it was noted that a subset of CD patients have seroreactivity to pANCA and phenotypically have “UC-like CD.” Continued research expanded the number of serologic markers that are associated with IBD and/or have the capacity to better differentiate CD and UC. The resulting set of serologic markers that now compose the PROMETHEUS IBD sgi Diagnostic test are detailed below.

Yeasts

ASCA-IgA and -IgG

ASCA (anti-Saccharomyces cerevisiae antibodies) are antibodies directed against mannose sequences from phosphopeptidomannan comprising the cell wall of the baker’s and brewer’s yeast Saccharomyces cerevisiae. Candida albicans has also been shown to express ASCA epitopes on mannoproteins similar to that found in S cerevisiae. ASCA has been shown to correlate with C albicans colonization in healthy relatives of patients with CD but not in CD patients themselves. The prevalence of ASCA positivity is highest in CD patients, ranging from 35% to 76%, but also occurs in 5% to 15% of patients with UC and in up to 5% of healthy controls.

Bacterial Proteins

Anti-OmpC

OmpC (outer membrane porin C) is a bacterial antigen originally isolated from Escherichia coli. This antigen results in immunoglobulin G and A (IgG and IgA) antibody responses (anti-OmpC) in 37% to 55% of adults with CD. In a cohort of children with CD, 17% were found to have a seropositive response to anti-OmpC. In another evaluation of antibody responses in children and young adults (age ≤24.0 yr) with CD or UC, anti-OmpC positivity was found in 25% of patients with CD, 11% of patients with UC, and 5% of healthy controls. While anti-OmpC as a stand-alone marker lacks sensitivity in CD and UC, the addition of this marker to a panel that includes ANCA, ASCA-IgG, and ASCA-IgA improves sensitivity for IBD. Indeed, a four-marker diagnostic panel including anti-OmpC identified CD in 65% of children and UC in 74% of children, with a 94% specificity.

Anti-CBir1

The human intestinal tract is colonized by a vast assortment of commensal microbial species, while also being occasionally exposed to bacteria that are potentially pathogenic. Subsets of both commensal and pathogenic bacteria are motile as a result of the expression of one or more flagella. The primary structural component of bacterial flagella is the 35-50 kDa protein flagellin. The region of the flagellin molecule involved in its polymerization to flagella is highly conserved among various bacteria. Flagellin has been found to be a major target of both innate and adaptive immune responses that are associated with IBD.

In 2004, Lodes and colleagues used serologic expression cloning to search for bacterial antigens relevant to IBD and identified flagellins as among the immunodominant antigens of the microbiota. The specific flagellin expression clone, CBir1, was subsequently noted to induce a strong antibody response (anti-CBir1) in four genetically distinct mouse models of IBD. In humans, these researchers noted significantly higher levels of anti-CBir1 in patients with CD than in patients with UC and controls, suggesting that this response might be useful in the diagnosis of IBD and in the differentiation of CD and UC. Further research by Targan and colleagues found that anti-CBir1 was expressed in 50% to 55% of patients with CD. Controlling for the previously described microbial antigens ASCA (IgG and IgA), anti-OmpC, and anti-I2 (antibody to the I2 bacterial sequence found in Pseudomonas fluorescens), these researchers found that anti-CBir1 was independently associated with CD (P<0.001) and its expression level was unrelated to any one of the other four individual antimicrobial antibodies associated with CD; as a result, anti-CBir1 reactivity defined another subpopulation of CD patients that was pathophysiologically distinct. Anti-CBir1 expression was also found in approximately 50% of CD patients who did not express ASCA; 40% to 44% of patients with CD whose sole antibody response was to pANCA were found to express anti-CBir1 compared with only 4% of patients with UC who were otherwise solely positive for pANCA. Furthermore, anti-CBir1 expression was found in 40% of patients who lacked a serologic response to any of the other antigens studied. More recently, Markowitz and colleagues investigated immune responses in 705 children, age 0 to 15 years, with CD. This group found that 52% were seronegative for pANCA, ASCA-IgA and -IgG, and anti-OmpC. This percentage of seronegative patients fell to 27% when assessment of anti-CBir1 was added (P<0.001), thus identifying a significant subset of children with CD who were otherwise seronegative.
Anti-Fla-X and Anti-A4-Fla2

In their research, Lodes and colleagues cloned a second reactive flagellin (Fla-X) that was highly homologous to CBir1 (83.5% similarity at the NH2 conserved domain) and, similar to the findings with CBir1, strong immune reactivity directed toward the NH2 terminal end of Fla-X was noted in both a colitic mouse model and in patients with CD. The level of response to Fla-X observed in sera from patients with CD was significantly higher than that found in sera from normal controls and patients with UC. Given that more than 50% of the UC sera tested came from patients with a Truelove and Witts severity index indicative of moderate to active disease (ie, >7), these antiflagellin responses have potential not only in the diagnosis of IBD but also in the discrimination of UC and CD.

Since prior phylogenetic analyses have linked the Clostridium phylogenetic cluster XIVa to the origin of flagellins, Duck and colleagues investigated this cluster and characterized a number of flagellated bacteria from it. These investigators found that the A4 bacterial strain expresses a flagellin, A4-Fla2, which has a very similar amino acid sequence to the flagellin Fla-X which patients with CD have demonstrated seropositivity. Subsequently, Schoepfer and colleagues assessed seroreactivity to A4-Fla2 and Fla-X in a population of well-characterized CD (n = 252) or UC (n = 53) patients and in healthy controls (n = 43). Seropositivity to A4-Fla2 and Fla-X was found in 59% and 57% of patients with CD, respectively, 6% each in patients with UC, and 0% and 2% of healthy controls, respectively. The serologic response generated against either flagellin in patients with CD was most frequently IgG, being found in 94% of those positive for anti-A4-Fla2 and in 93% of those positive for anti-Fla-X. An IgA response was found in <20% of CD patients for either flagellin. No correlation was found between disease duration and serologic response to A4-Fla2 or Fla-X. This research has underscored the dominance of flagellin as an antigen in CD. Given the slightly higher rate of seroreactivity to Fla-X and A4-Fla2 than to CBir1 noted in CD patients, these two flagellins may enhance the ability to diagnose CD over and above assessment of anti-CBir1.

Autoantibodies

ANCA, pANCA, and DNase-sensitive pANCA

pANCA (perinuclear antineutrophil cytoplasmic antibodies) are autoantibodies directed against a component of neutrophil granules. Distinct subgroups of ANCA have been noted with indirect immunofluorescence based on their individual staining pattern. The use of immunofluorescence has revealed a perinuclear pattern of staining (pANCA) in association with many different diseases including vasculitides and collagenous and eosinophilic colitides. The pANCA staining pattern has also been found in 30% to 83% of patients with UC, 6% to 20% of patients with CD, and up to 2.5% of healthy controls. Loss of this antigenic response after DNase digestion of neutrophils (ie, DNase-sensitive pANCA) appears to be a dominant characteristic of UC-specific pANCA, distinguishing the disorder from primary sclerosing cholangitis and type 1 autoimmune hepatitis.

Genetic Markers

GWAS methodology has revolutionized research into IBD. The GWAS approach is very effective in the detection of relatively common gene variants and forms the basis for fine-mapping of identified genes and research into their function. This has increased the knowledge of the various signaling pathways involved in the disorder(s) and also has implications for the development of future treatments. Genes that have been definitively associated with IBD, CD, or UC are listed in Figure 3 and the list is expanding rapidly.

ATG16L1

Defective bacterial handling has become one of the most consistent focuses in the study of CD pathogenesis. One gene that is related to the process of autophagy, ATG16L1, has been associated with CD, autophagy has many physiologic roles in health and disease, including acting as a “housekeeping” mechanism by which cells digest parts of their own cytoplasm for removal or providing an innate immune mechanism by which the host can combat intracellular bacteria. ATG16L1 was first identified by Hampe and colleagues as a susceptibility gene for CD in a GWAS of 19,779 non-synonymous single nucleotide polymorphisms (SNPs) present in 735 patients with CD and in 368 controls. Non-synonymous SNPs are SNPs that have different amino acids. Analysis showed that the marker rs2241880 encoded a threonine-to-alanine substitution at amino acid position 300 (T300A), which was correlated with the incidence of CD in one British and two German studies of CD. No correlation between the rs2241880 marker and UC was noted. Cummings and colleagues attempted to replicate the findings of Hampe and colleagues in a cohort of 648 CD cases, 677 UC cases, and 1190 controls that were well characterized. This group found that carriage of the G risk allele of rs2241880 was strongly associated with CD ($P=2.33\times10^{-7}$, odds ratio [OR] 1.45 [1.25 – 1.67]), thus reproducing the Hampe nonsNP GWAS findings. A recent meta-analysis of 25 studies (published up to June 1, 2009) evaluating the association of ATG16L1 and IBD found a positive association between the T300A polymorphism and susceptibility to CD (OR 1.32; 95% confidence interval [CI] 1.26 – 1.39; $P < .00001$). ATG16L1 was also associated with the risk of childhood-onset CD but not childhood-onset UC.

The same polymorphism was recently evaluated in a Spanish cohort comprising 557 CD and 425 UC patients compared with 672 ethnically matched controls and was found to be strongly associated with susceptibility to CD (OR 1.62) but showed no association with UC.
The STAT (signal transducers and activators of transcription)-Janus kinase pathway controls signal transduction between cell surface receptors and the nucleus. STAT3, an inducible DNA binding protein that binds to the interleukin (IL)-6 responsive element within the promoters of hepatic acute phase protein genes, is activated by a wide variety of cytokines and growth factors. Upon activation, STAT3 is phosphorylated, resulting in dimerization; the dimer migrates to the nucleus where it induces the expression of genes that have roles in many biologic functions including cell growth, antiapoptosis and proapoptosis, cell motility, negative feedback (suppression of cytokine production), regulatory cytokine production, and antibacterial activity.

It is well established that STAT3 is necessary for the signaling of proinflammatory cytokines such as IL-6, which is involved in the pathogenesis of both multiple sclerosis and IBD. STAT3 is also important for multiple aspects of the biology of Th17 cells, which, in turn, are critically important as mediators of a number of human inflammatory diseases. In mouse models, STAT3 plays distinct roles in both innate and acquired immunity. STAT3-mediated activation of acquired immune response plays a pathogenic role in

**Figure 3.** Genes Associated with Inflammatory Bowel Disease, Crohn’s Disease, and Ulcerative Colitis (Adapted with permission from Thompson and Lees.

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<th>CD</th>
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*NColonic disease

**NFK2.3**

Signaling pathways that are critical to normal mammalian gut development are dysregulated in IBD. NFK2.3 (NK2 transcription factor-related, locus 3) is a member of the NKX family of homeodomain-containing transcription factors implicated in cell type specification and maintenance of differentiation in several types of tissue. NFK2.3 is expressed in small blood vessel endothelial cells and intestinal lamina propria mesenchymal cells. Evidence from mouse studies suggests that abnormal expression of NFK2.3 alters the migration of antigen-responsive lymphocytes in the gut and influences the inflammatory response in the intestines. Susceptibility for CD was demonstrated with the rs10883365 variant of NFK2.3 in multiple GWAS studies in which ORs between 1.3 and 1.5 were observed. Additionally, in the first non-synonymous SNP scan in UC, Fisher and colleagues found an association between the rs10883365 NFK2.3 variant and UC (\(P = 3.3 \times 10^{-4}\)). Meggys and colleagues later found this association in a group of Czech and Hungarian IBD patients (increased risk for UC, \(P = 0.003\) after Bonferroni correction; OR 1.36, 95% CI 1.13–1.63), demonstrating that NFK2.3 represents an IBD locus.

**STAT3**

The STAT (signal transducers and activators of transcription)-Janus kinase pathway controls signal transduction between cell surface receptors and the nucleus. STAT3, an inducible DNA binding protein that binds to the interleukin (IL)-6 responsive element within the promoters of hepatic acute phase protein genes, is activated by a wide variety of cytokines and growth factors. Upon activation, STAT3 is phosphorylated, resulting in dimerization; the dimer migrates to the nucleus where it induces the expression of genes that have roles in many biologic functions including cell growth, antiapoptosis and proapoptosis, cell motility, negative feedback (suppression of cytokine production), regulatory cytokine production, and antibacterial activity.

It is well established that STAT3 is necessary for the signaling of proinflammatory cytokines such as IL-6, which is involved in the pathogenesis of both multiple sclerosis and IBD. STAT3 is also important for multiple aspects of the biology of Th17 cells, which, in turn, are critically important as mediators of a number of human inflammatory diseases. In mouse models, STAT3 plays distinct roles in both innate and acquired immunity. STAT3-mediated activation of acquired immune response plays a pathogenic role in...
colitis by enhancing survival of pathogenic T cells and by inducing tumor necrosis factor alpha (TNF-α). In contrast, STAT3-mediated activation of innate response contributes to the suppression of colitis by enhancing the mucosal repair and induction of mucin production.65

Using GWAS, Barrett and colleagues found that the rs744166 variant of STAT3 was associated with CD susceptibility.66 This association was confirmed in a Spanish cohort of patients reported by Cenit and colleagues. This group analyzed polymorphisms in the STAT3 region (rs3809758/rs744166/rs1026916/rs12948909). The haplotype conformed by the risk alleles of each polymorphism was significantly associated with both CD (P=0.005; OR 1.25, 95% CI 1.06–1.46) and UC (P=0.002; OR 1.19; 95% CI 1.02–1.38).67

**ECM1**

One of the most intriguing concepts that has emerged regarding the genetics of UC has been its association with epithelial barrier genes.68 Intercellular junctions between intestinal epithelial cells play a crucial gatekeeping role in the gut by allowing the extraction of needed nutrients while keeping pathogens out of the systemic circulation. These tight junctions determine overall intestinal permeability. In IBD, epithelial permeability is enhanced and is a predictor of clinical relapse.69,70

In the first non-synonymous SNP scan for UC, Fisher and colleagues were able to identify a previously unknown susceptibility locus, ECM1, in UC.71 ECM1 is an epithelial barrier gene that encodes the glycoprotein ECM1 (extracellular matrix protein 1), which is expressed in the small and large intestines where it interacts with the basement membrane and inhibits matrix metalloproteinase 9. ECM1 also strongly activates nuclear factor-κB (NF-κB) signaling, which is a key component in a variety of regulatory pathways. Expression of ECM1 is upregulated in esophageal squamous cell carcinoma and colorectal cancer.72 While an association with the ECM1 variant has been found for UC, it was not observed when formally tested in an adequate sample of CD subjects, implying that ECM1 may provide a specific susceptibility association for UC alone.73

**Inflammatory Markers**

The presence of intestinal inflammation is a primary criterion for making the diagnosis of IBD. Under normal conditions, the intestinal mucosa is in a state of “controlled” inflammation that is regulated by a delicate balance between proinflammatory factors and anti-inflammatory factors. In IBD, immune response activation causes the release of inflammatory cytokines (eg, TNF-α) and growth factors (eg, vascular endothelial growth factor [VEGF]) into gut tissues causing gut inflammation and injury.74,75 Inflammatory characteristics shared between patients with IBD and those with other chronic immune disturbances include immune activation, the infiltration of leukocytes into tissues, and an increased vascular density.76

**Intercellular Adhesion Molecule 1 and Vascular Cell Adhesion Molecule 1**

Dense inflammatory infiltrates, including monocytes, lymphocytes, and neutrophils, characterize IBD and are seen in distinct distributions in both CD and UC.77 Infiltration of these cells is stimulated both by various cytokines and by the interactions that occur between adhesion molecules expressed on inflammatory cells in the circulation and their integrin receptors on localized target cells.77

Intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are cytokine-inducible cell surface glycoproteins that belong to the immunoglobulin supergene family.78 Soluble ICAM and VCAM are also detected in serum and are believed to arise from proteolytic cleavage of the cell surface molecules. ICAM-1 is expressed at low levels on endothelial, epithelial, and other cells as well as on lymphocytes and monocytes.79 The transcription of ICAM-1 is upregulated by proinflammatory cytokines.79,80 In inflamed tissues, increased expression of ICAM-1 on endothelial cells promotes the recruitment of inflammatory cells expressing its ligands (any substance that binds specifically and reversibly to another chemical entity to form a larger complex), leukocyte function antigen-1, or macrophage activation complex-1, thus propagating the inflammatory process. In the rat, interferon-γ-induced expression of ICAM-1 has been noted to be higher in deeper mucosa/submucosa and muscle layer microcirculations than in the superficial mucosa—layers of the gut involved in the characteristic pathology of CD—where it may contribute to leukocyte-endothelial interactions.81

The expression of VCAM-1 is found on activated endothelial cells, macrophages, dendritic cells, and fibroblasts. Ligands for VCAM-1 include the integrins α4/β4 (very late antigen 4) and α4/β7, which are present on monocytes, lymphocytes, and eosinophils. Upon binding to its ligands, VCAM-1 modulates leukocyte adhesion to endothelial cells and migration to sites of inflammation.82,83

Jones and colleagues investigated circulating concentrations of ICAM-1 and VCAM-1 in 43 patients with IBD (22 with CD and 21 with UC) compared with healthy controls (n=90).77 Circulating
median levels of ICAM-1 were significantly higher in patients with active CD vs controls (273 vs 168 ng/mL; \( P < .003 \)). Median circulating VCAM-1 concentrations were significantly higher in active vs inactive UC patients (165 vs 117 U/mL; \( P < .005 \)) and were also significantly elevated compared with VCAM-1 concentrations in patients with active CD (124 U/mL; \( P < .02 \)) and in healthy controls (50 U/mL; \( P < .0001 \)).\(^7\) It should be noted that increased levels of ICAM and VCAM are not specific for IBD and can be found in other inflammatory, infectious, and neoplastic diseases.\(^\text{84} \)

**Vascular Endothelial Growth Factor**

Angiogenesis, the growth of new blood vessels from preexisting vasculature, is important in embryogenesis, tissue growth, wound healing, and ovulation. Angiogenesis plays a key role in pathologic processes as well, including cancer, ischemic cardiovascular disease, diabetic retinopathy, and in chronic inflammatory diseases such as IBD.\(^\text{85-87} \)

VEGFs represent a family of factors that mediate angiogenesis. Stimulation of angiogenesis is known to occur as a result of hypoxia or mechanical shear stress and stretch. Inflammation that underlies the pathology of IBD may promote angiogenesis in a number of ways. Many inflammatory cytokines upregulated in IBD are proangiogenic including IL-17 and TNF-\( \alpha \).\(^\text{88,89} \) Inflamed tissue, such as that seen in IBD, is often hypoxic,\(^\text{90} \) and this hypoxia can induce angiogenesis through upregulation of factors such as VEGF, basic fibroblast growth factor-1, TNF-\( \alpha \), and hypoxia-inducible factor-1.\(^\text{91,92} \) Neovascularization may also be stimulated by extravasated fibrinogen.\(^\text{93} \) Angiogenic factors produced by macrophages, mast cells, lymphocytes, and fibroblasts can stimulate growth of blood vessels.\(^\text{93} \) Additionally, shear stress on the endothelium related to increased blood flow itself may stimulate angiogenesis.\(^\text{54,56} \) Several studies have demonstrated that circulating levels of VEGF are elevated in patients with IBD compared with healthy or disease controls and that VEGF levels correlate with disease activity in IBD.\(^\text{96-101} \)

**C-Reactive Protein**

CRP, predominantly produced in liver cells, is one of at least 40 proteins that participate acutely in the inflammatory response. Normally, the amount of CRP produced by hepatocytes is low (<1 mg/L), but following an acute stimulus such as inflammation, the production of CRP is rapidly increased under the influence of IL-6, TNF-\( \alpha \), and IL-1\( \beta \).\(^\text{102} \) While the function of CRP in vivo is not completely understood, CRP is a useful marker for detecting infections and inflammation due to its rapid rise and short half-life (~19 h) and for assessing the effect of therapy on the underlying disease, as resolution of the stimulus triggering its production normalizes CRP levels.\(^\text{102,103} \)

Shine and colleagues were the first to show that a CRP increase can be used to differentiate IBD from functional bowel disorders. In 82 patients with chronic abdominal symptoms, 19 were diagnosed with CD, 22 with UC, and 41 with a functional bowel disorder. All of the 19 patients with CD and 59% of the 22 patients with UC showed increases in CRP compared with none of the 41 patients with functional symptoms.\(^\text{104} \) Schepfer and colleagues found that CRP had a 64% sensitivity and a 92% specificity in discriminating IBD (n=64) from IBS (n=30).\(^\text{105} \) A much larger study performed by Henriksen and colleagues in Norway provided CRP data on 454 patients with UC and 200 patients with CD, which was measured at diagnosis, with follow-up at 1 and 5 years.\(^\text{103} \) Patients with CD were noted to have a stronger mean CRP response when compared with those with UC at diagnosis (CD 51 mg/L vs UC 18 mg/L; \( P < .001 \)), at 1 year (CD 51 mg/L vs UC 18 mg/L; \( P < .001 \)) and at 5 years (CD 13 mg/L vs UC 6 mg/L; \( P < .001 \)) after diagnosis. Additionally, these investigators found that CRP levels at the time of diagnosis were related to the extent of disease in patients with UC, but no such association was found in CD.\(^\text{103} \)

**Serum Amyloid A**

Serum amyloid A (SAA) is a family of four proteins, including the acute phase proteins SAA1 and SAA2, whose primarily hepatic expression is markedly increased in response to a variety of inflammatory stimuli,\(^\text{106,107} \) much like CRP. As SAA has a physiologic level that is 10 times higher than that for CRP, it may allow for easier detection of slight elevations.\(^\text{108} \) Its longer half-life (24 hours) compared with that of CRP (19 hours) may make SAA more sensitive as an acute phase reactant. In contrast to differential CRP levels that have been seen in CD versus UC (see above), Niederau and colleagues found that SAA levels were similarly elevated in patients with both forms of IBD.\(^\text{109} \) Jijon and colleagues have demonstrated that SAA activates the NF-\( \kappa \)B signaling pathway, highlighting the proinflammatory capacity of SAA.\(^\text{110} \) NF-\( \kappa \)B is known to be one of the major regulatory components involved in the dysregulation of cytokine production and signaling mechanisms by intestinal epithelial cells, lymphocytes, and macrophages that has been implicated in the pathogenesis of IBD.\(^\text{111} \)

A summary of the new serologic, genetic, and inflammatory biomarkers that have been included in the new PROMETHEUS IBD sgi Diagnostic test can be found in Table 1.
### Table 1. New Serologic, Genetic, and Inflammation Biomarkers Included in PROMETHEUS IBD sgi Diagnostic Test

<table>
<thead>
<tr>
<th>Serologic Markers</th>
<th>General Prevalence</th>
<th>Role/Function</th>
<th>Effect</th>
</tr>
</thead>
</table>
| Anti-Fla-X and anti-A4-Fla2 | ● 57% of CD patients\(^{44}\) (anti-Fla-X)  
● 59% of CD patients\(^{44}\) (anti-A4-Fla2) | ● Fla-X\(^{38}\) and A4-Fla2\(^{42}\) are flagellins  
● Flagellin is the primary structural component of bacterial flagella, used for motility  
● Serve as immunodominant antigens in the microbiota\(^{38}\) | ● Valuable for the diagnosis of IBD, especially for the more precise discrimination of CD from UC |

<table>
<thead>
<tr>
<th>Genetic Markers</th>
<th>General Prevalence</th>
<th>Role/Function</th>
<th>Effect</th>
</tr>
</thead>
</table>
| ATG16L1 associated with CD | ● OR 1.45\(^{50}\) | ● Elongates membranes that form autophagosomes in autophagy\(^{50}\) | ● May decrease ability to remove intestinal microbes\(^{51}\)  
● Increases production of inflammatory cytokines from macrophages\(^{51}\) |
| ECM1 associated with UC | ● OR 1.3–1.4\(^{57}\) | ● Interacts with basal membrane, inhibits MMP9 proteins, and activates signaling of proinflammatory cytokine pathway NF-κB\(^{46}\) | ● Exhibits defective barrier function\(^{46}\)  
● Causes thickening and scarring of mucus membranes\(^{46}\)  
● Associated with defects in gut development, abnormal tissue architecture, abnormalities in migration and segregation of B and T cells\(^{57}\) |
| NKK2.3 associated with CD and UC | ● OR 1.2–1.6\(^{57}\) | ● Encodes transcription factor that functions in cell specification and maintenance of tissue differentiation\(^{46}\) | |
| STAT3 associated with CD and UC | ● OR 1.18\(^{46}\) | ● Plays a role in the Th17-dependent autoimmune process | ● Expansion and overactivity of Th17 helper cell cytokines can lead to intestinal inflammation\(^{112}\) |

<table>
<thead>
<tr>
<th>Inflammation Markers</th>
<th>General Prevalence</th>
<th>Role/Function</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>● Elevated in some patients with CD and UC and in distal colon tissue(^{75})</td>
<td>● Proangiogenic factor that causes vasoconstriction through nitric oxide release and increased permeability, endothelial cell proliferation, and directed migration and differentiation(^{75})</td>
<td>● Extensive transmural injury including edema, loss of goblet cells, decreased mucus production, crypt cell hyperplasia, and erosions and ulcerations(^{75})</td>
</tr>
</tbody>
</table>
| ICAM and VCAM | ● Expressed in high levels in IBD patients\(^{76}\) | ● Promotes recruitment of inflammatory cells\(^{76}\)  
● Involved in slow rolling of lymphocytes and monocytes during inflammatory cell recruitment\(^{75}\) | ● Initiation of leukocyte migration and local tissue inflammation\(^{77}\)  
● Circulating levels correlate well with disease activity\(^{77}\) |
| CRP | ● Elevated in the context of inflammation  
● CRP response may be higher in CD vs UC patients\(^{103}\) | ● Acute phase reactant that increases markedly when stimulated by inflammation\(^{102}\) | ● Can be used to differentiate IBD from functional bowel disorders\(^{104}\)  
● Levels correlate with extent of disease in UC\(^{103}\) |
| SAA | ● More sensitive but less specific than CRP in acute-phase monitoring of IBD\(^{113}\) | ● Promotes leukocyte chemotaxis, cellular adhesion  
● Increases cytokine production and metalloproteinase secretion in various cell types\(^{110}\) | ● May directly influence the development of inflammation in the intestine\(^{119}\) |
**Development and Validation of the Prometheus IBD sgi Diagnostic Test**

The **PROMETHEUS IBD sgi Diagnostic Algorithm**

The IBD sgi Diagnostic test algorithm was developed using a computational method called “Random Forest.”

The algorithm was developed using a total of 1,520 well-characterized samples from IBD and non-IBD patients from 50 centers in North America. A total of 1,083 samples were used to train the algorithm, and an independent cohort of 437 samples was used to validate and determine the performance of the test.

The algorithm uses measurements of 17 biologic markers (blood proteins and genes) to first predict whether a patient has IBD. Then, if the patient is predicted to have IBD, the algorithm examines the marker measurements to differentiate CD from UC.

The training and validation cohorts used to develop the PROMETHEUS IBD sgi Diagnostic test are detailed below in **Table 2**. “GI controls” were subjects who had non-IBD gastrointestinal disease.

**Table 2. Patient Allocation for the Training and Validation Phases of Development of the PROMETHEUS IBD sgi Diagnostic Test**

<table>
<thead>
<tr>
<th>Disease Diagnosis</th>
<th>Training Cohort n (%)</th>
<th>Validation Cohort n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohn’s Disease</td>
<td>419 (39)</td>
<td>153 (35)</td>
</tr>
<tr>
<td>Ulcerative Colitis</td>
<td>227 (21)</td>
<td>101 (23)</td>
</tr>
<tr>
<td>GI Controls</td>
<td>314 (29)</td>
<td>123 (28)</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>123 (11)</td>
<td>60 (14)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1083 (100)</strong></td>
<td><strong>437 (100)</strong></td>
</tr>
</tbody>
</table>

**Receiver Operator Characteristics (ROC) Analysis**

The accuracy of any diagnostic test is dependent on how well the test separates patients with a specified condition versus those without the condition. In **Figure 4**, the ROC curves corresponding to each individual marker of the new PROMETHEUS IBD sgi Diagnostic test are presented. In this figure, AUC represents the performance of the test. If an AUC value of 1 is obtained, this is representative of a “perfect” test to discriminate between those with and those without disease. An AUC of 0.5 represents a discriminatory performance that is equal to that achieved with random chance.

When the patterns of all biomarkers are combined and examined by the PROMETHEUS IBD sgi Diagnostic test algorithm, the performance of the test improved significantly compared with the performances of the individual markers alone, as illustrated in **Figure 5**.

**Figure 5.** The corresponding positive and negative predictive values (PPV and NPV, respectively) are listed in **Table 3**. When IBD is highly suspected, the PPV with the PROMETHEUS IBD sgi Diagnostic test is 98%. When IBD suspicion is low, the NPV with the PROMETHEUS IBD sgi Diagnostic test is 95%.

**Figure 4. Receiver Operator Characteristics (All Individual Markers)**
Table 3. Positive and Negative Predictive Values for the PROMETHEUS IBD sgi Diagnostic Test

<table>
<thead>
<tr>
<th>Prevalence, %</th>
<th>IBD, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive predictive value (PPV)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>56</td>
</tr>
<tr>
<td>50</td>
<td>88</td>
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<tr>
<td>85</td>
<td>98</td>
</tr>
<tr>
<td>Negative predictive value (NPV)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>95</td>
</tr>
<tr>
<td>50</td>
<td>77</td>
</tr>
<tr>
<td>85</td>
<td>37</td>
</tr>
</tbody>
</table>

**Conclusion**

The diagnosis of IBD and the differentiation between CD and UC can pose a number of challenges in the clinical setting. Invasive radiographic and endoscopic diagnostic investigations are not without complications, and there is a subset of patients for whom these tests do not result in a definitive diagnosis. Diagnostic delays may adversely affect the management of IBD and illustrate the need for diagnostic accuracy. Ongoing research into the underlying pathophysiology of IBD has helped to identify more markers, including serologic, genetic, and inflammatory markers, that may prove to be a useful, noninvasive adjunctive diagnostic tool when IBD is suspected. Helping to provide actionable diagnostic information has the potential to allow for personalized treatment planning and better patient outcomes.
References


53. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007;447(7145):661-678.


Prometheus diagnostic services provide important information to aid in the diagnosis and management of certain diseases and conditions. How this information is used to guide patient care is the responsibility of the physician.

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