

**REVIEW****NONINVASIVE METHODS IN EVALUATION OF INFLAMMATORY BOWEL DISEASE: WHERE DO WE STAND NOW? AN UPDATE**Cansel Turkey,<sup>I</sup> Benan Kasapoglu<sup>II</sup>

doi: 10.1590/S1807-59322010000200015

Turkey C, Kasapoglu B. Noninvasive methods in evaluation of inflammatory bowel disease: where do we stand now? An update. Clinics. 2010;65(2):221-31.

The inflammatory bowel diseases, consisting of Crohn's disease, ulcerative colitis and indeterminate colitis, are distinguished by idiopathic and chronic inflammation of the digestive tract. The distinction between inflammatory bowel diseases and functional bowel disorders, such as irritable bowel syndrome, can be complex because they often present with similar symptoms. Rapid and inexpensive noninvasive tests that are sensitive, specific and simple are needed to prevent patient discomfort, delay in diagnosis, and unnecessary costs. None of the current commercially available serological biomarker tests can be used as a stand-alone diagnostic in clinics. Instead, these are used as an adjunct to endoscopy in diagnosis and prognosis of the disease. Along these lines, fecal lactoferrin and calprotectin tests seem to be one step further from other tests with larger number of studies, higher sensitivity and specificity and wider availability.

**KEYWORDS:** Inflammatory bowel disease; Diagnosis; Serology; Fecal markers.

**INTRODUCTION**

The inflammatory bowel diseases (IBD), consists Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC) which are distinguished by idiopathic and chronic inflammation of the digestive tract. These diseases have been shown to result from an aberrant innate and acquired immune response to commensal microorganisms in genetically susceptible individuals.<sup>1</sup> Currently, the incidence of IBD is increasing worldwide, especially in Northern Europe and North America. Ethnic origin, lifestyle, presence of susceptibility regions on at least 12 chromosomes and geographical factors play a central role in the epidemiology of these diseases.<sup>2,3</sup>

The distinction between IBD and functional bowel disorders, such as irritable bowel syndrome (IBS), can be complex since they often present with similar symptoms, including abdominal distention, pain and diarrhea, and therefore, invasive and expensive tests may be necessary. The diagnoses of IBDs depend on the clinical findings after radiological, endoscopic and histological examinations. Although the division between UC and CD is generally clear, indeterminate colitis is present in 10-20% of patients with isolated colitis.<sup>4</sup> Noninvasive tests for both the diagnosis and follow-up of IBD have gained increasing attention. Rapid and inexpensive noninvasive tests that are sensitive, specific and simple are necessary to prevent patient discomfort, delay in diagnosis and unnecessary costs. The biomarkers of IBD, including serological tests, fecal markers and genetically predisposed gene polymorphisms, are tools for disease diagnosis, estimation of activity, follow-up and disease prognosis.<sup>5-7</sup> Moreover, in conjunction with the development of imaging techniques, such techniques as imaging biomarkers with ultrasound, magnetic resonance imaging (MRI), X-ray, computer tomography (CT), position emission tomography (PET)

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Received for publication on August 28, 2009

Accepted for publication on November 05, 2009

and single photon emission computed tomography (SPECT) are also defined.<sup>8</sup>

Therefore, the purpose of the present study was to critically review the current literature on the diagnosis and follow-up of inflammatory bowel diseases. We systematically searched Medline and the Cochrane Database, with no language restrictions, for studies of humans on the topic of IBD diagnosis that were published between January 1960 and August 2009. The key words *inflammatory bowel diseases, Ulcerative colitis, Crohn's disease, fecal calprotectin, lactoferrin, serology* and their equivalent Medical Subject Heading terms were used.

## SEROLOGICAL MARKERS

Serological testing has been used for many years in the diagnosis of IBDs. Serological biomarkers are primarily produced upon intestinal exposure to normal commensal bacteria<sup>9,10</sup> and might reflect a dysregulated immune inflammatory response.<sup>11,12</sup> Most of the major serological biomarkers utilized in IBD clinics are antibodies to microbial antigens, including yeast oligomannan (anti-*Saccharomyces cerevisiae*, ASCA), bacterial outer membrane porin C (OmpC), *Pseudomonas fluorescens* bacterial sequence I2 (anti-I2) and, most recently, bacterial flagellin (CBir 1).<sup>13</sup>

All of these antibodies are predominantly found in CD but are not found in UC, except ASCA, which is identified in 5% of UC patients. On the other hand, the human antibody, perinuclear antineutrophil cytoplasm antibody (pANCA) is considered to be an autoantibody, although the specific antigenic stimulation for its production remains imprecise. pANCA has currently been found in up to 70% of patients with UC and in up to 20% of patients with CD.<sup>14</sup>

Five new anti-glycan antibodies anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA), anti-mannobioside IgG (AMCA) and antibodies against chemically synthesized ( $\Sigma$ ) two major oligomannose epitopes, Man  $\alpha$ -1,3 Man  $\alpha$ -1,2 Man ( $\Sigma$ Man3) and Man  $\alpha$ -1,3 Man  $\alpha$ -1,2 Man  $\alpha$ -1,2 Man ( $\Sigma$ Man4) are recognized recently.<sup>13,15</sup> Since these new biomarkers have been shown to be present only in IBD, they might signify an intestinal inflammation that is specific to UC or CD. Moreover, these antibodies have been primarily studied in CD and have a high specificity but poor sensitivity.

Joossens et al. investigated 86 families from Belgium and Northern France to test whether a combination of CD-associated genes and/or antibody responses to microbial antigens might be valuable in identifying healthy relatives at risk. Genetic (NOD2, NOD1, TLR4, CARD8) and new serologic markers (ASCA, ACMA, ALCA, ACCA,

ASigmaMA, OmpC, CBir1, I2) were analyzed in all of the subjects. After a follow-up of 54 months, the authors found that there was an additive risk for CD in subjects from multi-case families per additional affected relative and per additional positive antibody, and this was independent of NOD2 genetic marker.<sup>16</sup> These new antibodies might be important in complicated disease phenotype and might predict the need for surgery.

Recently, Mokrowiecka studied 125 IBD patients (71 UC, 31CD and 23 IC) and 45 patients with functional intestinal disorders to determine the accuracy of pANCA and ASCA in patients with IBD subgroups. In UC patients, the prevalence of pANCA was 68%, which was significantly higher than in CD (29%). ASCA were found significantly more often in CD (80.6%) than in UC patients (26.8%). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of pANCA for UC diagnosis were 68%, 84%, 75% and 78%, respectively, and of ASCA for CD diagnosis were 81%, 78%, 45.5% and 95%, respectively. Moreover, the combined use of these two markers provided changes in diagnostic accuracy, such that for pANCA+/ASCA- in UC the sensitivity, specificity, PPV and NPV of results were 42%, 100%, 100% and 43%, respectively, and for pANCA-/ASCA+ in CD the results were 52%, 98.6% 94% and 82%, respectively. The authors concluded that the specificity of these combined serological markers tended to be higher than their sensitivity, and thus, these markers are more useful in the differentiation of IBD subtypes than in screening the population.<sup>17</sup>

Anand et al. evaluated 98 adults with IBD and found that ASCA and pANCA had a 32% sensitivity and 100% specificity for Crohn's disease, while there was a 50% sensitivity and 90% specificity for UC.<sup>18</sup>

Interestingly, in another study, the presence of ASCA was found to be associated not only with the existence of Crohn's disease but also with markers of disease severity and oral involvement.<sup>19</sup>

Two novel immunoglobulin A (IgA) cell wall polysaccharide antibodies, anti-laminarin (anti-L) and anti-chitin (anti-C), were analyzed during the diagnosis and phenotype differentiation of Crohn's disease and UC. A cohort of 818 individuals with IBD (517 CD and 301 UC) were analyzed for seven anti-glycan antibodies (gASCA (anti-*Saccharomyces cerevisiae*) IgG, gASCA IgA, anti-chitobioside (GlcNAc(beta1,4)GlcNAc(beta)), anti-laminaribioside (Glc(beta1,3)Glb(beta)), anti-mannobioside (Man(alpha1,3)Man(alpha)), anti-L and anti-C) and for pANCA.<sup>20</sup> The authors found that all of the glycan markers were specific for and more prevalent in CD than in UC and, additionally, that gASCA IgG and IgA best differentiated CD from UC, followed by anti-L. The authors concluded that

anti-L and anti-C improved the ability to differentiate between CD and UC and that these antibodies were independently associated with a more aggressive CD phenotype. Chen et al. described the use of a whole *E. coli* proteome microarray as a novel high-throughput approach to screen and identify new serological biomarkers for IBD. With the use of protein arrays containing 4,256 *E. coli* K12 proteins, Chen et al. have identified novel sets of serological biomarkers for the diagnosis of IBD that have a >80% overall accuracy and sensitivity in differentiating CD from UC.<sup>21</sup>

It is important to keep in mind that the diagnostic value of serological biomarkers can show a discrepancy among different ethnic or geographic groups. For instance, both ASCA and pANCA were found to be less sensitive in Chinese and Japanese patients, while the positivity of pANCA was shown to be higher in Mexican-American UC patients.<sup>22,23</sup>

It is also essential to emphasize that none of the current commercially available serological biomarker tests can be used alone as a diagnostic in clinics. Instead, they are used in addition to endoscopy in diagnosis and prognosis of the disease. Whether or not serologic markers have a role in screening for IBD remains controversial. However, due to the generally low sensitivity and specificity of these markers for distinguishing IBD from non-IBD, they are generally not recommended for use as a screening test. As a consequence, specific and sensitive IBD serologic biomarkers are desired, as well as future studies to evaluate the efficacy of current and newly identified biomarkers.

## BLOOD INFLAMMATORY MARKERS

Erythrocyte sedimentation rate (ESR), white blood cell count (WBC) and C-reactive protein (CRP) are known to be good predictors of disease activity in IBD. CRP, with its short half-life, becomes rapidly elevated soon after the onset of the inflammatory process and decreases after its resolution. Moreover evaluating CRP is simple, easily available and inexpensive. ESR is also inexpensive and easily available, but since it has a longer half-life it differs from CRP and causes a prolonged latency period after changes in IBD activity. In clinical practice, because ESR, WBC and CRP are non-specific, they sometimes are not helpful for the differential diagnosis and follow-up of IBD.<sup>24,25</sup>

In addition, ESR has been found to be more reliable to be correlated with the disease activity.<sup>26</sup> The pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6, and IL-8) are also found to be elevated in IBD patients.<sup>27</sup> However, these are not widely available and are not specific for intestinal inflammation.

## FECAL MARKERS

Fecal markers comprise a heterogeneous group of substances that either pour out from, or are generated by, the inflamed intestinal mucosa.<sup>28</sup> The fecal excretion of **Indium 111-labeled leukocytes** is considered to be the gold standard fecal marker of inflammation, with a sensitivity of 97% for the diagnosis of IBD.<sup>29</sup> Even though the use of radio-labeling techniques remains very important for research studies, they are not recommended for routine use due to high cost, exposure to radiation and the need for 4 days of fecal collection.

Fecal levels of **Alpha1 1-antitrypsin**, which is a protease inhibitor produced by the liver, macrophages and intestinal epithelium, are a useful indicator of IBD. Random levels of fecal **Alpha1-antitrypsin levels** are revealed to be useful in measuring CD activity, while testing a 72-h fecal clearance of **Alpha1-antitrypsin** is a useful method for quantification of intestinal protein loss.<sup>30,31</sup> Although fecal  $\alpha$ 1-antitrypsin has been generally accepted as a useful marker of IBD, it is not routinely available and cost-effective.

Fecal excretion of another serum anti-proteinase, **alpha2-macroglobulin**, is also increased in IBD patients. The levels of alpha2-macroglobulin in the feces have a positive relationship with the activity index in CD but not in subjects with UC.<sup>32</sup>

The neutrophil-derived proteins, **lysozyme, myeloperoxidase, calprotectin, lactoferrin, and PMN-elastase**, are generally elevated in the feces of IBD patients.<sup>33-39</sup> However, fecal lactoferrin and calprotectin are more appropriate for the differentiation of chronic IBD from IBS, and their increased levels show a positive relationship with the severity of inflammation. Some recent studies that deal with the relationship of fecal markers in IBD are summarized in Table 1.<sup>48-52, 59, 66, 68-74</sup>

### Fecal Lactoferrin

Lactoferrin is an 80-kDa iron-binding glycoprotein and a major component of the secondary granules of polymorphonuclear neutrophils. In intestinal inflammation, leukocyte infiltration of the mucosa causes a rise in lactoferrin concentration in the feces. Lactoferrin has antibacterial activity and is resistant to proteolysis in the feces. Lactoferrin can be detected using simple and inexpensive techniques since it has an excellent stability in the feces for 4 days since a commercial ELISA has been developed and is now widely available. A negative fecal lactoferrin test simply means that there is an absence of significant neutrophilic intestinal inflammation.<sup>40, 41</sup>

**Table 1** - Some recent studies about the fecal markers in the evaluation of IBD

Study	Aim	Patient	Result	Conclusion
<b>Erbayrak et al<sup>48</sup></b>	To investigate the role of FC in evaluating IBD activity	65 IBD and 20 IBS patients	ESR, CRP, and FCP values were higher in the IBD patients than in the control group, while the hgb level was lower in the IBD group. No statistically significant differences in FCP levels were detected between UC and CD patients.	FC was found to be strongly associated with colorectal inflammation indicating organic disease.
<b>Schoepfer et al<sup>49</sup></b>	To evaluate the correlation between endoscopic disease activity and FC, CAI, CRP, and blood leukocytes in UC	134 UC patients and 48 controls	FC levels were significantly lower in UC patients with inactive disease. The overall accuracy for the detection of endoscopically active disease was 89% for FC, 73% for CAI, 62% for elevated CRP, and 60% for leukocytosis.	FC was the only marker that reliably discriminated inactive from active disease, emphasizing its usefulness for activity monitoring.
<b>Eder et al<sup>50</sup></b>	To evaluate the diagnostic utility of the assessment of FC concentration in patients with CD.	31 CD and 12 IBS patients	Mean FC concentration in CD group was statistically higher than among IBS patients. There was a positive correlation between FC concentration and CRP, and negative--with hemoglobin concentration.	The assessment of FC concentration may be useful in differential diagnoses of CD and monitoring patients with CD.
<b>Gisbert et al<sup>51</sup></b>	To determine the role of FC and FL in the prediction of IBD relapses	89 CD, 74 UC patients	Sensitivity and specificity to predict relapse of IBD for FC (>150 microg/g) and FL were 69% and 69%, and 62% and 65%, respectively.	FC and FL determination may be useful in predicting impending clinical relapse--in both CD and UC patients.
<b>D'Inca et al<sup>52</sup></b>	To assess the role of calprotectin tests in predicting clinical relapse in IBD patients.	97 UC and 65 CD	A significant correlation emerged between a positive FC test and the probability of relapse in UC patients. In CD patients, only cases of colonic CD showed a significant correlation between a positive FC test and the probability of relapse.	Measuring calprotectin may help to identify UC and colonic CD patients at higher risk of clinical relapse.
<b>Jeffrey et al<sup>59</sup></b>	To investigate fecal tumor M2-PK in the differentiation of functional from organic bowel disease.	94 controls and 105 outpatients of whom 14 were diagnosed with organic bowel disease later	Sensitivity and specificity, for diagnosis of organic bowel disease were 93%, 92% for FC and 67%, 88% for tumor M2-PK, respectively.	Tumor M2-PK is able to differentiate organic from functional bowel disease but has a lower sensitivity, specificity, and predictive value than FC.
<b>Wagner et al<sup>66</sup></b>	To evaluate FC as a marker of treatment outcome of relapse of IBD and, to compare FC with fecal myeloperoxidase (MPO) and fecal eosinophil protein X (EPX)	27 UC and 11 CD patients	A normalised FC level at the end of the study predicted a complete response in 100% patients, whereas elevated FC level predicted incomplete response in 30%. Normalised MPO or EPX levels predicted a complete response in 100% and 90% of the patients, respectively. However, elevated MPO or EPX levels predicted incomplete response in 23% and 22%, respectively.	A normalised FC level has the potential to be used as a surrogate marker for successful treatment outcome in IBD patients. FC and MPO provide superior discrimination than EPX in IBD treatment outcome.
<b>Lamb et al<sup>68</sup></b>	To evaluate FC and FL in identifying CD recurrence in symptomatic patients after ileocaecal resection	117 CD patients	In patients with mild to moderately clinically active disease, FC and FL identified individuals with and without recurrent IBD. Faecal markers were more accurate at predicting clinical disease activity than CRP, platelet count or endoscopic appearance.	FC and FL are non-invasive tests that can help to identify disease recurrence in symptomatic postoperative patients.
<b>Ashorn et al<sup>69</sup></b>	To identify new noninvasive test combinations for characterization of IBD in children and adolescents by comparing serological responses to microbial antigens.	73 children who underwent endoscopies because of suspicion of IBD and IBD was diagnosed in 60 patients (18 CD, 36 UC, 6 IC).	FC levels were elevated more frequently in IBD patients (89% vs 9%). ASCA antibodies were detected in 67% of patients with CD, The combination of the measurements of FC and serological responses to microbial antigens (ASCA, I2, and OmpW) identified 100% of CD patients (specificity 36%) and 89% of UC patients (specificity 36%).	Increased levels of serological responses to microbial antigens (ASCA, I2, and OmpW) and FC are evident in both CD and UC patients. The combination of these markers provides valuable, noninvasive tools for the diagnosis of IBD.

**Table 1** - Some recent studies about the fecal markers in the evaluation of IBD (cont.)

Study	Aim	Patient	Result	Conclusion
Walkiewicz et al <sup>70</sup>	To compare FC levels in IBD and healthy controls, to correlate FC levels with clinical disease activity,	32 IBD patients and 34 healthy controls	The IBD group had higher FC levels compared with control. Among those with clinical relapse, 90% had FC levels more than 400 mug/g in CD. Eighty-nine percent of CD encounters with FC levels less than 400 mug/g remained in clinical remission.	Among children with CD and in remission, FC levels may be useful in predicting impending clinical relapse.
Xiang et al <sup>71</sup>	To investigate possibility and clinical application of FC in determining disease activity of UC	66 UC and 20 control patients	The FC concentration in the patients with active UC was significantly higher than inactive UC which was higher than the control group. There was a strong correlation between the FC concentration and the endoscopic gradings for UC .	FC can reflect the disease activity of UC and can be used as a rational marker for intestinal inflammation in clinical practice.
Ho et al <sup>72</sup>	To investigate FC as a biomarker in predicting the clinical course of acute severe UC	90 patients with acute severe UC requiring intensive in-patient medical therapy	FC was significantly higher in patients requiring colectomy, with a trend toward significance when comparing corticosteroid nonresponders and responders, as well as between infliximab nonresponders and responders	FC levels are dramatically elevated in severe UC. This biomarker can predict response to first or second-line medical therapy in this setting.
Sipponen et al <sup>73</sup>	To study the correlation of FC and FL with simple endoscopic score for Crohn's disease (SES-CD) and histology.	24 CD patients with 87 ileocolonosopies	In ileocolonic or colonic disease, both FC and FL correlated significantly with colon SES-CD and colon histology. In patients with normal FC or FL levels, endoscopic and histology scores were significantly lower than in those with elevated concentrations.	In ileocolonic and colonic disease, endoscopic score SES-CD and histological findings correlated significantly with FC and FL.
Langhorst et al <sup>74</sup>	To compare the performance of FL, FC, polymorphonuclear neutrophil elastase (PMN-e), and CRP in patients with IBD to address whether these markers can differentiate IBD patients with endoscopically assessed inflammation;and they correlate with endoscopic severity of inflammation	54 IBS, 42 UC, 43 CD patients	UC or CD patients with active inflammation demonstrated significantly higher levels of FL, FC, and PMN-e in feces as well as serum-CRP when compared to patients with inactive inflammation and patients with IBS. FC showed the highest diagnostic accuracy in CD (81.4%), whereas FL was superior to the other markers in UC (83.3%). The comprehensive activity index yielded a further improvement of sensitivity and specificity, with a diagnostic accuracy of 95.3% for UC patients.	The fecal markers FL, FC, and PMN-e are able to differentiate active IBD from inactive IBD as well as from IBS.

FC: Fecal calprotectin, IBD:Inflammatory bowel disease, IBS: Irritable bowel syndrome, ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, UC: Ulcerative colitis, CD: Crohn disease, CAI: Clinical activity index, FL: Fecal lactoferrin, IC: Indetermined colitis.

Dai et al. studied a total of 177 fresh stool samples collected from 42 active UC, 17 inactive UC, 13 active CD, 5 inactive CD, 41 infectious bowel diseases, 25 IBS and 34 healthy volunteers to evaluate the relationship between fecal lactoferrin and intestinal inflammation by quantitative analysis. Fecal lactoferrin was found to be significantly higher in active IBD than in inactive IBD, IBS and infectious bowel disease. The sensitivity and specificity of fecal lactoferrin for UC were 92% and 88%, respectively, and for CD were 92% and 80%, respectively. As a result of this study, fecal lactoferrin was found to be a sensitive and specific marker in measuring the activity of IBD and a valid method for discriminating between inflammatory and non-inflammatory bowel diseases.<sup>42</sup>

Kane et al. compared 104 CD, 80 UC and 31 IBS patients with 56 healthy controls to determine the sensitivity and specificity of fecal lactoferrin concentrations for IBD or IBS. The study found that fecal lactoferrin was 90% specific for identifying inflammation in patients with active IBD, and elevated levels of lactoferrin were 100% specific in ruling out IBS.<sup>43</sup>

Schopper et al. studied 64 patients with IBD (36 CD, 28 UC), 30 with IBS and 42 healthy controls to determine the accuracy of fecal markers, CRP, blood leukocytes and antibody panels for discriminating IBD from IBS. In addition to CRP and blood leukocytes, blinded fecal samples were measured for calprotectin (PhiCal Tesr, ELISA), lactoferrin (IBD-SCAN, ELISA), Hexagon-

OBTI (immunochromatographic test for detection of human hemoglobin), and LEUKO-TEST (lactoferrin latex-agglutination test). Also, the blinded serum samples were measured for the ASCA (ELISA) and pANCA (immunofluorescence) antibodies. The authors found that fecal calprotectin and lactoferrin could accurately discriminate between IBD and IBS. Moreover, there was only a marginal improvement in diagnostic accuracy when ASCA and pANCA were also involved.<sup>44</sup>

Another study of 20 patients with IBS, 36 with IBD (24 CD, 12 UC) and 18 with other forms of colitis (8 infectious colitis, 5 ischemic colitis, 5 medication-induced colitis) was conducted to evaluate the accuracy of four different fecal markers in discriminating between IBS, IBD and other forms of colitis. In this study, blinded fecal samples were measured for calprotectin (with PhiCal-Test, ELISA), lactoferrin (with IBD-SCAN, ELISA), with Hexagon OBTI (immunochromatographic test for detection of human hemoglobin) and with LEUKO-TEST (lactoferrin latex-agglutination test). The overall accuracies for discriminating IBS from IBD or other forms of colitis were as follows: IBD-SCAN, 91%; PhiCal-Test, 89%; LEUKO-TEST, 92%; Hexagon OBTI, 91%; C-reactive protein, 89%; and blood leukocytes, 92%. The differentiation of IBD from other forms of colitis using fecal markers had an overall accuracy ranging from 43 to 50%. The feasibility of fecal sampling in outpatients was high, with an acceptance rate of 95%. In conclusion, the IBD-SCAN and PhiCal-Test had the best overall accuracy for the detection of colitis, followed by the LEUKO-TEST, Hexagon OBTI, C-reactive protein and blood leukocytes.<sup>45</sup>

Fecal lactoferrin might be a helpful noninvasive diagnostic tool for the detection of colitis; however, since it is unspecific, its role in the diagnosis and monitoring of IBD is still questionable. Further studies are necessary to determine its exact place in routine clinical practice.

### Fecal Calprotectin

Calprotectin is a calcium-binding protein that inhibits metalloproteinases, has antibacterial and antifungal activities and induces apoptosis in malignant and nonmalignant cell cultures.<sup>46</sup> Calprotectin constitutes 60% of neutrophil cytosolic proteins and is an abundant protein found in all body fluids in proportion to the degree of inflammation. Calprotectin has many clinical advantages. It is resistant to bacterial degradation in the gut and is stable in stool for up to one week at room temperature, allowing delays in transporting the sample to the laboratory. Furthermore, calprotectin can be readily quantified using ELISA. Notably, random stool samples of <5 g show calprotectin

concentrations equivalent to 24-hour homogenized specimens, demonstrating that calprotectin is uniformly scattered throughout the feces.<sup>47</sup>

Since calprotectin is primarily derived from neutrophils, its concentration is directly proportional with neutrophil migration toward the intestinal tract. Many studies have dealt with the role of calprotectin in IBD diagnosis and follow-up (Table 1). The leukocyte proteins calprotectin, lactoferrin, lysozyme, myeloperoxidase, and PMN-elastase were compared in fecal samples of three consecutive feces (e.g., three days) in 40 healthy persons, 39 patients with chronic IBD (21 with CD and 18 with UC) and 40 patients with IBS. From this comparison, levels of all of the fecal leukocyte markers in IBS were found to be in the range of healthy patients. Moreover, fecal PMN-elastase and calprotectin still differentiated between chronic IBD and IBS and still correlated with the severity of inflammation.<sup>34</sup>

In our study of 65 IBD patients (14 CD and 51 UC) and 20 outpatients diagnosed with IBS according to Roma II criteria, fecal calprotectin was found to be strongly associated with colorectal inflammation, indicating the presence of organic disease.<sup>48</sup>

Another study was conducted to evaluate the correlation between endoscopic disease activity and fecal calprotectin. The results of the Clinical Activity Index (CAI), CRP and blood leukocytes in 134 UC patients found that endoscopic disease activity correlated closest with the presence of calprotectin. The overall accuracy for the detection of endoscopically active diseases (score  $\geq 4$ ) was 89% for calprotectin, 73% for CAI, 62% for elevated CRP and 60% for leukocytosis. In conclusion, fecal calprotectin was the only marker that reliably discriminated an inactive disease from mild, moderate and highly active diseases, highlighting its usefulness for monitoring activity.<sup>49</sup>

In a different study of 31 patients diagnosed with CD, the mean calprotectin concentration in the CD group was statistically higher than that of the IBS patients. A concentration of 16.01 mg/l calprotectin had 67.7% sensitivity and 66.7% specificity in distinguishing between CD and IBS. In this respect, the assessment of fecal calprotectin concentration might also be useful for differentiating CD from IBSCD and IBS.<sup>50</sup>

Gisbert et al. followed up 163 patients (89 CD, 74 UC) for 12 months who had been in clinical remission for 6 months to determine the role of fecal calprotectin and lactoferrin in the prediction of IBD relapse. The authors reported that 26 patients (16%) relapsed during follow-up. Calprotectin concentrations in patients who had suffered a relapse were found to be higher than in patients who had not (239  $\pm$  150 versus 136  $\pm$  158  $\mu$ g/g;  $P < 0.001$ ). The relapse risk was higher in patients that had high (>150

µg/g) calprotectin concentrations (30% versus 7.8%;  $P < 0.001$ ) or positive lactoferrin (25% versus 10%;  $P < 0.05$ ). The sensitivity and specificity of fecal calprotectin (>150 µg/g) to predict relapse were 69% and 69%, respectively. The corresponding values for lactoferrin were 62% and 65%, respectively. As a result, it was concluded that the determination of fecal calprotectin and lactoferrin might be useful in predicting an impending clinical relapse, especially during the following 3 months of remission, in both CD and UC patients.<sup>51</sup>

Similarly, in another study with 97 UC and 65 CD patients in clinical remission, a significant correlation was found between a positive calprotectin test and the probability of relapse in UC patients ( $P = 0.000$ ). However, in CD patients, only cases of colonic CD had a significant correlation between a positive calprotectin test and the probability of relapse ( $P = 0.02$ ).<sup>52</sup> Although fecal calprotectin levels are considered to change with age, 50 µg/g of the suggested cut-off level is considered to be useful for all age groups over 4 years old.<sup>53</sup>

However, there are 4 main handicaps of fecal calprotectin to be kept in mind:

- In some studies, low-dose aspirin treatment did not increase fecal calprotectin levels, although the use of non-steroidal anti-inflammatory drugs (NSAIDs) might cause an increase in calprotectin levels due to NSAID-induced enteropathy in patients without IBD.<sup>54,55</sup>
- Any bleeding in the body over 100 ml, including menstrual bleedings, might increase fecal calprotectin levels.<sup>56</sup>
- Some authors suggest that, although fecal calprotectin is considered to be evenly distributed, factors other than disease might contribute to the significant intraindividual biological variations of it.<sup>57</sup>
- Since levels of fecal calprotectin increase in any condition that causes neutrophil migration to the gut, including neoplasms and infections, the sensitivity of fecal calprotectin is not as high as desired.

Fecal calprotectin is an easy, inexpensive, sensitive and specific way to evaluate IBD. Despite the fact that levels of fecal calprotectin have an important role in diagnosis, follow-up, prediction of relapses and assessment of response to treatment, it still has some disadvantages and can only be used as a complementary test.

### Fecal Pyruvate Kinase

The dimeric isoform of M2-pyruvate kinase (tumor M2-PK), suggested to be a marker of colorectal cancer, has also recently been suggested to be a marker of gastrointestinal inflammation.<sup>58</sup>

Jeffery et al. studied 105 gastroenterology outpatients with a possible diagnosis of organic bowel disease and 94 controls to investigate the role of fecal tumor M2-PK in the differentiation of functional disease from organic bowel disease. The sensitivity, specificity and positive and negative likelihood ratios for diagnosis of organic bowel disease were found to be, respectively, 93%, 92%, 11.6 and 0.07 for calprotectin, and, respectively, 67%, 88% 5.6 and 0.18 for tumor M2-PK. Calprotectin, in combination with tumor M2-PK, had a sensitivity of 64%, a specificity of 98% and likelihood ratios of 32 and 0.03. Tumor M2-PK was useful for the differentiation of organic disease from functional bowel disease but had a lower sensitivity, specificity and predictive value than calprotectin.<sup>59</sup>

The clinical value of fecal pyruvate kinase in IBD patients requires further study.

### Rectal Nitric Oxide

Nitric oxide (NO) is an endogenously produced gas with numerous physiological roles. In response to acute proinflammatory cytokines, leukocytes and epithelial cells express inducible nitric oxide synthase (NOS), which leads to the production and accumulation of significant quantities of NO.<sup>60</sup>

The level of rectal NO correlates with disease activity in IBD patients and it markedly decreases in response to anti-inflammatory treatment. This minimally invasive and rapid test is shown to be useful for discriminating between active bowel inflammation and IBS.<sup>61</sup> Reinders et al. also studied 23 healthy volunteers and 32 patients with IBD to compare calprotectin and rectal NO levels. These authors found that patients with IBD had greatly increased NO and calprotectin levels compared to healthy volunteers ( $p < 0.001$ ). Moreover, there was a weak correlation between rectal NO levels, disease activity and the number of loose stools in IBD patients (Spearman's rho 0.37 and 0.51, respectively;  $p < 0.05$ ); interestingly, there was no correlation between NO and calprotectin levels.<sup>62</sup>

Ljung et al. studied 22 UC and 24 CD patients to explore rectal nitric oxide (NO) as a biomarker for the treatment response in IBD. Patients with active UC and CD displayed markedly increased rectal NO levels compared to the controls. Rectal NO correlated weakly with disease activity in both UC and CD. Interestingly, the patients' refractory to steroid treatment only slightly increased NO levels compared to those with a therapeutic response. In this respect, the rectal NO level might be a useful biomarker for the treatment response in IBD, since low NO levels are predictive of a poor clinical response to steroid treatment.<sup>63</sup>

However, although rectal NO is a minimally invasive

test and more expensive than many other fecal tests. More studies are necessary to reveal the exact role of rectal NO levels in IBD patients.

### Fecal Myeloperoxidase

Myeloperoxidase, an enzyme that functions in the oxygen-dependent killing of microorganisms, is released from the primary granules of neutrophils during acute inflammation. The concentration of myeloperoxidase is also proportional to the number of neutrophils within that region.<sup>64</sup>

Silberer et al. compared five different leukocyte proteins, calprotectin, lactoferrin, lysozyme, myeloperoxidase and PMN-elastase and determined their levels by immunoassay in the feces of patients with IBD and IBS and of healthy persons. The areas under the ROC curves of PMN-elastase and calprotectin were not significantly different ( $p = 0.327$ ), whereas PMN-elastase or calprotectin vs. the other proteins were significantly different ( $p < 0.001$ ). The results suggest that fecal PMN-elastase and calprotectin are important for the differentiation of chronic IBD from IBS. The authors also found that PMN-elastase and calprotectin levels were correlated with the endoscopically classified severity of inflammation but not the myeloperoxidase.<sup>34</sup>

However, Peterson et al. found a relationship between fecal myeloperoxidase levels and the histological indices of disease activity in UC.<sup>65</sup>

Similarly, Wagner et al. showed that normalized MPO levels predicted a complete response to treatment in 100% of the patients, as did normalized fecal calprotectin levels. However, elevated MPO levels predicted an incomplete response in 23% patients.<sup>66</sup>

In this respect, myeloperoxidase might potentially be used as a surrogate marker for a successful treatment outcome in IBD patients, similar to calprotectin. Further investigations are necessary to identify the clinical role of fecal myeloperoxidase in IBD.

### Fecal Eosinophil Protein X

Eosinophil protein X (EPX) is released by activated eosinophil granulocytes, which are abundant in the mucosa in active IBD.<sup>67</sup> Fecal EPX levels are mainly studied as an indicator of the treatment outcome in relapses of IBD. Wagner et al. showed that normalized EPX levels have predicted a complete response to treatment in 90%; however, an incomplete response was predicted in 22% of the patients. In this respect, FC and MPO provide superior discrimination compared to EPX in IBD treatment outcome.<sup>66</sup> Moreover, fecal EPX levels are also beneficial complements to

endoscopic and histopathological evaluations in the daily care of patients with UC.<sup>65</sup> Still, more studies are necessary to reveal the clinical role of fecal EPX in IBD.

### CONCLUSION

Since inflammatory bowel diseases are chronic, fast, easily available and inexpensive noninvasive tests that are sensitive, specific and simple are necessary for diagnosis and follow-up. A differential diagnosis of organic and inorganic diseases is also important since they might have similar symptoms. Along these lines, fecal lactoferrin and calprotectin tests seem to be one step further from other tests with larger number of studies, higher sensitivity and specificity and wider availability.

#### Take-home points:

- None of the current commercially available serological biomarker tests can be used by themselves in clinics for diagnosis and follow up. Instead, the tests are used as an adjunct to endoscopy in diagnosis and prognosis of the disease.
- The erythrocyte sedimentation rate (ESR), white blood cell count and C-reactive protein (CRP) are good predictors of disease activity in irritable bowel diseases (IBDs). However, since they are non-specific, they are sometimes not helpful for the differential diagnosis and follow-up of IBD.
- Indium 111-labeled leukocytes are considered to be the gold standard fecal marker of inflammation, with a 97% sensitivity for the diagnosis of IBD. However, due to their high cost, the exposure to radiation and the need for prolonged fecal collections of 4 days, they are not recommended for routine use.
- Even though fecal  $\alpha 1$ -antitrypsin and alpha2-macroglobulin are generally accepted as useful markers of IBD, they are not routinely available or cost-effective.
- Fecal lactoferrin might be a helpful as a noninvasive diagnostic tool for the detection of colitis; however, since it is unspecific, its role in diagnosis and monitoring of IBD remains questionable. Fecal calprotectin is an easy, inexpensive, sensitive and specific method with which to evaluate IBD. Although levels of fecal calprotectin are important in all diagnoses, follow-ups, predictions of relapses and assessment of response to the treatment, it still can only be used as a complementary test.
- Tumor M2-PK differentiates organic disease from functional bowel disease but has a lower sensitivity, specificity and predictive value than does fecal calprotectin.
- Rectal nitric oxide is a minimally invasive test and is more expensive than many other fecal tests.



- Fecal myeloperoxidase and eosinophil protein X have potential as a surrogate marker for the determination of successful treatment outcomes in IBD patients, similar to calprotectin.
- Further studies are necessary to elucidate the exact role of fecal markers in IBD evaluation.

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