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Inflammatory Bowel Disease Biomarkers

Dandan Liu[1], **Varma Saikam**[1], **Katie A. Skrada**[1], **Didier Merlin**[2,3], **Suri S. Iyer**[1]

[1]788 Petit Science Center, Department of Chemistry, Georgia State University, Atlanta, Georgia 30302, USA.

[2]790 Petit Science Center, Institute for Biomedical Sciences, Georgia State University, Atlanta, Georgia 30302, USA.

[3]Atlanta Veterans Medical Center, Decatur, GA, USA.

Abstract

Inflammatory bowel disease (IBD) is characterized as chronic inflammation in the gastrointestinal tract, which includes two main subtypes, Crohn's disease (CD) and ulcerative colitis (UC). Endoscopy combined with biopsy is the most effective way to establish IBD diagnosis and disease management. Imaging techniques have also been developed to monitor IBD. While effective, the methods are expensive and invasive, which leads to pain and discomfort. Alternative non-invasive biomarkers are being explored as tools for IBD prognosis and disease management. This review focuses on novel biomarkers that have emerged in recent years. These serological biomarkers and miRNAs could potentially be used for disease management in IBD thereby decreasing patient discomfort and morbidity.

Keywords

Inflammatory bowel disease; Biomarker; Diagnostics; Therapeutics; non-invasive

Introduction:

Inflammatory bowel disease (IBD) is characterized by chronic inflammation in the gastrointestinal tract.[1] The specific cause of IBD is unknown, although several factors contribute to the disease, such as genetic predisposition, dysregulated immune responses and environmental factors.[2] 6.8 million individuals were estimated to be diagnosed with IBD globally in 2017, which is a significant increase from the 3.7 million individuals diagnosed in 1990.[3] IBD can be classified as ulcerative colitis (UC) or Crohn's disease (CD) and is typically diagnosed by endoscopic biopsy. However, 10–15% of patients diagnosed with IBD are categorized as indeterminate colitis (IC) patients, a form that is difficult to distinguish between UC and CD.[4] This indeterminate form can develop into UC or CD as symptoms increase in severity. UC is normally associated with continuous colon inflammation, starting in the rectum and extending to the proximal segments of the colon, mainly affecting the inner epithelial layer of the colon.[5] It mostly affects adults in the 30–40

siyer@gsu.edu, dmerlin@gsu.edu.

age group and can be debilitating.[6] UC can further be classified into three types based on the extent of colonic involvement: proctitis, left-side colitis, and extensive colitis (Figure 1). Proctitis affects 30–60% of patients and symptoms include rectal bleeding, tenesmus, and urgency. Left-side colitis affects 16–45% of patients, and these patients have proctitis symptoms in addition to diarrhea and abdominal cramping. The most traumatic form of UC is extensive colitis that affects 15–35% of patients. This form can have all symptoms of left-side colitis in addition to constitutional symptoms, fatigue, and fever.[7,8]

In contrast to UC, CD can affect any portion of the gastrointestinal tract from the mouth to the anus, generally afflicting individuals between 15 and 30 years old.[9] CD is non-continuous and can affect all the layers of the intestinal wall. CD patients have symptoms that include abdominal pain, fever and clinical signs of bowel obstruction or diarrhea with passage of blood or mucus (Figure 1).[9] Although some therapeutic drugs are effective for UC and CD, other therapies are more specific to UC or CD. UC and CD patients have different clinical treatment courses and differ in the preferred timing of surgery.[4] It is important to accurately differentiate between UC and UC in patients.

In addition to gastrointestinal symptoms, 25–40% of patients with IBD can express extraintestinal manifestations, which mainly affect joints, skin, liver and eyes.[10] Manifestations involved in joints include peripheral arthritis, which mainly affects the large joints of arms and legs;[11] axial arthropathy, which mainly results in pain in the lower back, hips, and buttocks;[12] and ankylosing spondylitis, which can lead to vertebrae fusion over time.[13] Manifestations that are involved in the patients skin include: erythema nodosum, pyoderma gangrenosum, and aphthous ulcers.[14–16] Manifestations involved in the liver include primary sclerosing cholangitis, which is characterized by inflammation and scarring of bile ducts.[17,18] Manifestations involved in eye include: episcleritis, which can cause eye redness, swelling, and inflammation, as well as uveitis.[19,20]

In this review article, we will discuss current diagnostics and potential biomarkers for diagnosis and disease maintenance in IBD. Other reviews[21,22] have explored diagnosis of IBD. However, this review includes detailed diagnosis of IBD, and novel biomarkers discovered recently. Compared to the other review papers of IBD, we have provided detailed discussion about each biomarker, including structure, function, pathway, and concentration ranges in healthy subjects and IBD patients. Furthermore, we introduced next-generation biomarkers that could be very valuable for researchers in this field.

Therapeutics:

Anti-inflammatory therapies are considered as the first step in treatment for IBD; these include 5-aminosalicylates (5-ASA) and corticosteroids.[23] Several studies have demonstrated that 5-ASA is effective in IBD by activating γ-form peroxisome proliferator-activated receptors (PPAR-γ).[24–26] Due to the poor toleration of sulfasalazine, a variety of mesalamine derivatives have been developed to deliver 5-ASA, such as Olsalazine[27], Balsalazide[28] and Pentasa[29]. 5-ASA is effective for treatment of active CD but is not recommended for IBD maintenance. For treatment of UC, 5-ASA is effective for the induction and maintenance of remission in mild to moderate active UC.[23]

Corticosteroids can bind to the intracellular glucocorticoid receptor and enter the nucleus to produce anti-inflammatory effects.[30] Common types of corticosteroids include prednisone, methylprednisolone, hydrocortisone and budesonide.[31] Prednisone and methylprednisolone are used to treat moderate to severe cases of IBD;[32] hydrocortisone is used for a short treatment course;[33] budesonide is used to treat moderate cases of CD.[34]

Immunosuppressive agents are mainly used when patients do not show improvement with 5-ASA and steroids.[35] Immunomodulators include 6-mercaptopurine (6-MP), azathioprine (AZA), methotrexate (MTX) and cyclosporine (CSA). 6-MP is active form of AZA, both of them can inhibit lymphocyte proliferation, which might lead to anti-inflammatory effects through suppression of natural kill cell and T cell activity.[35] MTX is a folic acid antagonist, which can inhibit interleukin (IL)-1 and suppress T cell function. It has been reported that MTX was used for the induction and maintenance of CD. [36] CSA is a calcineurin inhibitor, which can form a complex with cyclophilin to block the phosphatase activity of calcineurin, resulting in the decrease of inflammatory cytokines produced from T cell.[37] It is effective for the induction and maintenance of UC, but no convincing data for CD treatment have been provided.

Tumor necrosis factor-alpha (TNF-α) inhibitors, also called biologics, are available as injectables to treat IBD. Human TNF is a family of proteins and receptors that involved in immune regulation.[38] Anti-TNF-α can neutralize TNF-α, by blocking proinflammatory signaling mechanisms. TNF-α inhibitors such as infliximab, adalimumab, certolizumab pegol are available as therapies to treat IBD.[39] Most recently, melatonin and metabolites from commensal microbiota are being considered as potential therapies to inhibit inflammation in IBD. [40–43] Current treatments for IBD are summarized in Table 1.

Diagnostics:

Endoscopy Procedures with Biopsy

The most effective method to definitively establish a diagnosis for UC and CD is through the combination of endoscopy procedure with a biopsy of the targeted area. This procedure, although invasive and uncomfortable, accurately differentiates UC from CD and is used to monitor disease severity.[44] Ileocolonoscopy with collection of multiple biopsy specimens is a well-established first line diagnostic in CD.[45] The features of endoscopic finding of CD includes the discontinuous chronic and patchy distribution of inflammation with skip lesions.[46] During endoscopic procedures in patients with CD, inflammation can present in different ways: erythema, altered vascular pattern, friability, granularity and small discrete superficial and aphthous ulcers. As inflammation becomes more severe, deep, serpiginous and linear ulcerations and a “cobblestone” appearance of the inner wall can develop.[47] Anatomical criteria of severe inflammation in CD includes deep ulcerations across the muscle layer, mucosal detachments, or ulceration limited to the submucosa but extending to more than one-third of a defined colonic segment.[48] Endoscopy in patients with UC exhibit inflammation that is continuous throughout the colon. Mild inflammation in UC presents as edema, erythema, and abnormal vascularity. Moderate inflammation can have a “wet sand-paper” appearance, erosions, superficial ulcers, and friability. Severe inflammation in UC appears as confluent ulcerations, worsened friability of epithelium, and has the

potential to develop into spontaneous bleeding.[49,50] The endoscopy might also reveal rectum sparing or patchy rectal inflammation after treatment. To obtain a reliable diagnosis, a minimum of two biopsies from five sites around the colon, including rectum and ileum is obtained.[51,52] The microscopic features identified in CD include discontinuous chronic and patchy chronic inflammation, discontinuous crypt, and granulomas. The features above combined with irregular villous architecture can be used to identify the biopsy samples taken from the ileum.[46] UC biopsy samples are characterized by diffused mucosal granularity, edema and erythema.[53] Obtaining a full set of biopsy samples from the colon can improve the diagnostic yield for both UC and CD, which also might reveal inflammation not seen in endoscopy. Though this method may be time-consuming and invasive, the use of endoscopy combined with biopsy sampling has the advantage to diagnose IBD at an early stage.

Several tools have been developed to evaluate the disease activity and severity of CD. Crohn's Disease Activity index (CDAI)[23] is commonly used to evaluate the symptoms of CD and Crohn's Disease Endoscopic Index of Severity (CDEIS)[35] is commonly used to evaluate the severity of CD with endoscopy. The index is composed of eight factors, each factor is adjusted with weighting factor (Table 2). In contrast, CDEIS consists of four parameters: deep ulcerations, superficial ulcerations, surface involved by disease, surface involved by ulcerations. Each parameter is evaluated in five segments of the colon. Depends on the score, it can be classified as remission (< 3), mild activity (3–9), moderate activity (9–12) and severe activity (>=12).

Imaging

Numerous studies have investigated less-invasive imaging methods to diagnose IBD such as magnetic resonance imaging (MRI), computed tomography (CT), ultrasonography (US), scintigraphy and positron emission tomography (PET).[54–56] Imaging plays an important role in early diagnosis of IBD. It can provide evidence of abnormal bowel in patients with suspected IBD, particularly CD, and further examine where abnormalities are distributed in the gastrointestinal tract.[57] A meta-analysis study by Horsthuis, K. et al., discussed 33 studies that compared the precision of US, MRI, CT and scintigraphy imaging to diagnose patients with suspected IBD.[58] The sensitivity and specificity for each method were 90% and 96% for US, 93% and 93% for MRI, 84% and 95% for CT, 88% and 85% for scintigraphy, for UC and CD, respectively. CT and MRI are used primarily to view the small intestine. Both methods can establish the locations and activity of IBD through wall thickness and increased intravenous contrast enhancement. Imaging with US and scintigraphy can be used to take images of the colon. MRI and US are the most accurate, and in contrast to CT, do not use ionizing radiation, which can be harmful to patients.[59] Considering the relapsing nature of IBD, frequent reevaluation for patients is necessary and therefore, MRI and US is generally preferred for diagnosis and follow-up evaluation of IBD.

Biomarkers

Since endoscopy and other methods discussed in the previous section require invasive and uncomfortable procedures, efforts to diagnose and monitor inflammation are being evaluated. Non-invasive or minimally invasive self-testing methods are ideal as it decreases hospital visits. To this end, considerable effort has been directed towards the development

of discovering novel biomarkers to diagnose and monitor IBD. A biomarker is defined as a substance that can be objectively measured or evaluated from a tissue or biofluid present in the targeted specimens.[60] Several IBD specific biomarkers (Table 3) are discussed in detail in the following sections.

A. Serological biomarkers.

1. C-reactive protein (CRP).: CRP is considered as one of the most important proteins present in acute inflammation, and was first discovered in 1930 in patients with pneumococcal pneumonia.[61] The production of CRP is amplified in response to most forms of inflammation, infection, and tissue damage in endothermic animals.[62] Human CRP consists of 187 amino acids and is composed of five identical non-glycosylated polypeptide subunits (Figure 2).[62] From the pentagonal appearance in electron microscope images, CRP became part of the “pentraxin family” of calcium-dependent ligand-binding plasma proteins.[62,63] Each subunit present in CRP has the ability to bind two calcium ions which allows it to bind to a variety of ligands.[63] The ligand for which CRP has the highest affinity for is phosphocholine (PC), which is involved in the phospholipids in cell membranes and lipoproteins.[64] CRP has distinct functional activities in its native and non-native pentameric conformations. The native form is binding to PC and in contrast, the non-native form binds to the factor H.[65]

The promoters present in CRP take on a “lectin-fold” formation, which are composed of a two-layered β-sheet with flattened jellyroll topology.[61,64] The two calcium ions present are bound 4 Å apart by protein sidechains at the ligand binding site on the concave side of the subunit, designated B. The other side of each subunit, designated A, consists of a single α helix and has a marked furrow, which is made of positively charged residues lining the outside and negatively charged residues on the inside.[64] CRP is synthesized by hepatocytes, and is rapidly produced following the release of pro-inflammatory cytokines, such as interleukin-6 (IL-6) by macrophages and T cells.[62,66] The half-life of plasma CRP remains constant at 19 hours under all conditions, making the rate of synthesis the determining factor of concentration; therefore the levels of CRP directly represent the strength of pathological stimulation in the body. There are two principle methods to detect CRP; through the use of enzyme-linked immunosorbent assays (ELISA), or by rate nephrometry in a clinical setting.[47] In healthy adults the median concentration of CRP is ~1 mg/L.[62] However, following acute stimulation, CRP levels can increase substantially ranging from 50 – 500 mg/L. Examples of major CRP acute-phase responses include: bacterial and fungal infections, allergic reactions, inflammatory diseases, necrosis, trauma, or cell malignancies. When CRP is released, it is deposited into the damaged tissue and activates complement, which has pro-inflammatory effects and promotes beneficial scavenging functions. This process enhances tissue injury and can lead to more advanced disease.[62,64] For this reasoning, circulating CRP levels has been proposed as a biological marker in several diseases and is highly relevant in measuring severity and extent of IBD.[62]

The correlation between elevated CRP levels and IBD in patients has been investigated. Under normal bowel conditions, the concentration of serum CRP detected is 1–3 mg/L. In patients with mild to moderate inflammation, the CRP levels can increase to 50–100 mg/L

in 4–6 hours.[67,68] A population-based data study showed that at the time of diagnosis, the median CRP concentration for CD patients is 40 mg/L and in UC patients is 20 mg/L.[69] To further examine the correlation of CRP levels to IBD, a study by Solem, C. et al. was performed to examine the clinical, endoscopic, and histologic activity of both CD and UC. The results revealed that patients with CD had elevated levels of CRP. They were most associated with active irritable bowel during colonoscopy (OR, 3; 95% CI, 1–18), as well as histologically severe forms of the disease (OR, 10; 95% CI; 1–104). In UC patients, CRP levels were only associated with histologically severe forms ($P = 0.029$).[66] In more recent years, several studies have been conducted to compare the levels of CRP in patients treated with the drug infliximab. Louis, E. et al. compared the CRP levels in CD patients before and after treatment;[70] while Iwasa, R. et al. compared them in patients with UC before and after treatment.[71] The results indicated that the concentration levels of CRP in patients that positively responded to infliximab had decreased significantly.[70,71] These results, demonstrate that CRP is an effective tool in monitoring inflammation flareups and treatment efficacy in IBD.

2. Erythrocyte sedimentation rate (ESR).: ESR determination is a test that can indicate acute inflammation in patients in a simple and inexpensive manner. The test measures the rate of erythrocytes falling through a vertical column of anticoagulated blood under the influence of gravity.[72,73] The two main factors that determine ESR are the degree of red blood cell aggregation and hematocrit, also known as packed cell-volume. Red blood cell aggregation is affected by the proteins present in blood plasma.[74] In cases of positive inflammatory response, the erythrocytes will fall at a faster rate leading to more aggregation. This positive correlation allows this test to be used to measure the inflammatory activity that is caused by IBD.[75] Turner, D. et al. evaluated the use of ESR to monitor UC in patients, and also compared it to use of CRP.[75] They found the median ESR values for different severity levels of UC to be as follows: around 17 mm/h in patients in remission from UC, 26, 37 and 39 mm/h in patients with mild, moderate and severe UC, respectively. A meta-analysis performed by Holtman, G. et al. showed that the sensitivity and specificity values for ESR to diagnose IBD were 66% and 84%, respectively.[76] While ESR is less accurate than CRP for the diagnosis of IBD, it still proves to be a useful tool in combination with CRP. However, ESR could potentially be used to monitor inflammation flareups.

3. ASCA, p-ANCA, and other antibody markers.: Anti-Saccharomyces cerevisiae antibodies (ASCA) and perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) have been characterized as serological markers for inflammation for several years. They were first discovered in UC patients in 1990.[77] ASCAs were detected in the serum of 50 – 60% of patients with CD; and had diagnostic values for sensitivity and specificity of 67% and 92%, respectively.[78] P-ANCAs were detected in the serum of 60 – 70% of UC patients, with 15% of CD patients presenting antibodies.[79] Most studies used enzyme-linked immunosorbent assay (ELISA) to detect ASCA and p-ANCA; a sample was considered positive when a concentration of >15 U/ml was detected. Indirect immunofluorescence assay (IIF) has also been used to measure p-ANCA to differentially diagnose patients with CD or UC; a sample was considered positive when >24 U/ml.[80] Mokhtarifar, A. et al. tested patients at a one-year follow up exam, for ASCAs and p-ANCAs to re-examine their diagnoses of UC

or CD.[80] The results showed that ASCA+/p-ANCA- can diagnose CD with a sensitivity of 67% and specificity of 78%; while, ASCA-/p-ANCA+ can diagnose UC with a sensitivity of 78% and specificity of 67%, respectively. Other antibody markers have shown positive correlations with IBD patients; these include anti-outer-membrane porin C (OmpC) and anti-Cbir1 antibodies. These have been positively detected in around 50–55% of CD patients, 5–10% of UC patients, and 5–8% of healthy subjects.[81–84] Combining multiple antibodies in a single test is preferred as it decreases false positives and negatives. In one study, a group of four genetic markers (ATG16L1, NKX2–3, ECM1, and STAT3), five inflammatory markers (CRP, SAA, ICAM-1, VCAM-1, and VEGF), and two serological markers (A4-Fla2 and FlaX) were selected for the diagnosis of IBD in combination with a six serological marker panel (ASCA-IgA, ASCA-IgG, ANCA, pANCA, OmpC, CBir1).[85] The results of the combination test were compared to the results from the six serological marker panel for comparison.[85] The outcome showed that with this extended marker panel, discrimination area under the curve of IBD versus non-IBD increased to 0.87 (95% CI, −0.4 – 0.4) from 0.80 (95% CI, −0.5 – 0.5; $P < 0.001$). In addition, the discrimination area under the curve for differential diagnosis of UC from CD increased to 0.93 (95% CI, −0.4 – 0.4) from 0.78 (95% CI, −0.6 −0.6; $P < 0.001$), indicating that multiple biomarkers offer a higher level of precision.

4. Nitric oxide (NO).: NO is a stable, yet mildly reactive free radical and gaseous signaling molecule present in mammalian cells plays a vital role in the regulation of various physiological and pathophysiological responses.[86] Some of these processes include vascular homeostasis, neurotransmission of the central nervous system and in peripheral nerves, as well as hemostasis and host defense.[86,87] NO can have varying effects on the body based on the chemical environment and levels of NO present, sometimes resulting in opposing outcomes.[87] At low levels of NO and in consequence of transition metal interactions, the signaling and protective actions of NO progress, which have the ability to terminate free radical pathways that cause harm. Contrarily, at higher levels of NO, this mediator has cytotoxic effects that are important for the microbicidal activity of macrophages but can further damage tissue in areas of injury or inflammation. The synthesis of NO is performed through the oxidation of amino acid L-arginine by a family of enzymes designated as the nitric oxide synthases (NOSs).[88] There are three NOS isoforms: neuronal (nNOS), expressed in the brain and peripheral nervous system; endothelial (eNOS), expressed in endothelial cells; and inducible (iNOS), which is expressed in response to microbial products such as interleukin-1 (IL-1) or tumor necrosis factor- α (TNF-α).[86] While inflammatory cytokines such as these have been positively correlated to IBD, iNOS activity has also been positively demonstrated in active UC.[89] The possibility of NO as a mediator in the inflammatory processes of IBD has shown significant interest.[88] Several manifestations of IBD have been found to directly or indirectly correspond with NO, such as vasodilation and increased vascular permeability. A study by Avdagić, N. et al. investigated the potential of serum NO as biomarker to diagnose UC and CD.[90] The result showed the level of serum NO was statistically different between UC patients, CD patients, and healthy controls. The median NO concentrations in UC patients, CD patients, and healthy controls were 15.3 μM, 14.5 μM, and 13.3 μM, respectively. With a cut-off of 17.4 μM, the sensitivity and specificity of NO to differentiate between active and inactive UC patients were both 100%. And with a

cut-off of 14 μM, the sensitivity and specificity of NO to differentiate between active and inactive CD patients were 88% and 69%, respectively. These results indicate that serum NO could be a potential biomarker for IBD.

5. Cytokines.: Tumor necrosis factor-α (TNF-α) was first identified in the 1970's as a soluble cytokine capable of significant cytotoxic activity against tumor cell lines, and is released upon activation of the immune system.[91] TNF-α is expressed mainly by activated macrophages and lymphocytes, and has two forms: transmembrane and soluble form. The transmembrane form is processed by TNF-α-converting enzyme (TACE) to become soluble TNF-α, which is cleaved and can bind to receptors present on tissues.[92] However, both forms are important for pro-inflammatory activity, and have vital roles in the pathogenesis of chronic inflammatory diseases. TNF-α possesses multiple therapeutic roles in the human body including: immunostimulant, infection resistance, tumor resistance, and sleep regulation.[93] However, the main role of this protein is to mediate resistance against infections through the activation of neutrophils, platelets, macrophages and natural killer cells. Furthermore, excessive amounts of TNF-α can result in toxicity in the host, as this protein can induce necrosis and apoptotic cell death in the body. The effects of TNF-α are carried out when the trimer (Figure 3) protein binds and forms clusters with high-affinity receptors TNF-R1 and TNF-R2 on cell membranes.[92,93] The trimer conformation of TNF-α is important to form stable complex with its receptors by crossing the energy barriers and induce signal transductions on cell surface.[94] Several receptors that bind TNF-α have been identified and form a large superfamily of type I transmembrane glycoproteins. Once TNF-α is bound to its receptor, depending on the type of cell membrane, it will play part in numerous physiological and pathological responses.[92]

TNF-α can be found in elevated levels in the GI tract of colitis patients and has a major role in mucosal inflammation.[92,95] Anti-TNF agents have been used to counteract IBD inflammation and has proven to be an effective treatment for some individuals. Avdagić, N. et al. investigated the concentration levels of TNF-α present in the serum of IBD patients compared to healthy controls using ELISA.[95] The concentration levels of serum TNF-α present in UC patients, CD patients, and healthy controls were found to be 29.3, 29.5, and 28.9 pg/ml, respectively. The concentrations did not show significant differences between patients with IBD and healthy controls in relation to disease activity. However, this could be due to the method of testing and the existing difficulties in measuring the changes in serum TNF- α concentrations.[95,96] Another study by Komatsu, M. et al. tested the concentrations of TNF- α using highly sensitive immuno-PCR, which resulted in concentrations that were significantly different between IBD patients and healthy controls.[96] Results for median TNF-α concentrations were 7.6, 12.7 and 0.02 pg/ml in patients with UC, CD and healthy controls. This study was the first to result in strikingly higher concentrations of TNF- α in IBD patients compared to healthy individuals, presumably due to the higher sensitivity of immune-PCR assay. TNF- α in the inflamed mucosa of IBD patients' needs further exploration but should be considered as a potential biomarker for its strong correlation to inflammation in the body.

There are several other cytokines indicate the inflammation in IBD, such as interleukin-6 (IL-6), IL-8, IL-10, IL-17 and IL-22.[97–99] ILs are a group of cytokines first discovered

express in leukocytes and there are more than 50 ILs in human.[100] IL-10 is exhibits significant concentration differences in IBD patients.[101–103] IL-10 was first identified in 1989 as a cytokine synthesis inhibitor produced by a subset of murine T lymphocytes, T-helper-2 (Th2) cells.[104] It is an 18.5 kD cytokine with broad immunoregulatory activity.[105] IL-10 is a dimer, each monomer consists of six helices (Figure 4). Each IL-10 molecule can bind with two molecules of IL-10 receptor.[106] IL-10 is an immunoregulatory cytokine and inhibits the production of proinflammatory cytokines, such as TNF-α, IL-1, IL-6 and IL-12.[107–109] It is also a growth and differentiation factor for B cells, thymocytes and mast cells, and it plays an significant role in preventing inflammatory and autoimmune pathologies.[110,111] IL-10 is mainly secreted by monocytes and release to serum and stool.[112] Kühn, R. et al. found that IL-10 deficient mice develop chronic intestinal inflammation.[113] Therefore, IL-10 knockout mice models are used for studying colitis. Mitsuyama, K. et al. compared the concentration of IL-10 in active UC, CD patients and healthy subjects with ELISA.[97] Kucharzik, T et al.[114] revealed human IL-10 serum levels were significantly increased in patients with active UC (144+34 pg/ml), $P<0.001$) and in active CD (132+32pg/ml, $P<0.001$) compared with healthy controls (44±9–5pg/ml). These results demonstrated that the concentration of IL-10 increased in active UC and CD patients, indicating that IL-10 is a suitable biomarker for IBD.

6. ST2.: Suppression of tumorigenicity 2 (ST2) was first discovered in 1989. ST2 belongs to interleukin 1 receptor family.[115] Two forms of the receptor exist: a transmembrane form and a circulating, soluble form that can be detected in serum samples. ST2 is mainly expressed in cardiac fibroblasts and cardiomyocytes in response to injury or stress. However, non-myocardial sources of ST2 are known, and are associated in inflammatory and immune processes.[116] The transmembrane form is structured like any type I interleukin-1 receptor: a transmembrane segment, with an extracellular domain of three linked immunoglobulin-like motifs, and an intracellular interleukin-1 receptor cytoplasmic domain. The circulating form does not posess the transmembrane portion or the cytoplasmic domains, but consists of unique nine amino acid C-terminal sequence.[115] ST2 is a receptor for interleukin-33 (IL-33), a cytokine secreted in response to tissue or cell damage.[116] The processes of IL-33 and ST2 in the pathogenesis of IBD and chronic inflammatory processes has garnered significant interest due to the high expression of ST2 levels in inflamed mucosa.[117] Overexpression of ST2 has been positively identified in correlation with UC. Boga, S. et al. set out to investigate the endoscopic, clinical, and histopathological assessment for serum ST2 levels in patients with UC and CD, in comparison to healthy controls.[117] 143 IBD patients participated in this study, 83 UC subjects and 60 CD subjects, along with 50 healthy controls. ST2 serum levels were detected using ELISA testing; and endoscopic disease activity was measured by the CD activity index (CDAI) and the clinical colitis activity index (CCAI). The median concentrations of serum ST2 in UC patients, CD patients, and healthy controls were 54 pg/ml, 64 pg/ml, and 31 pg/ml, respectively. This study found that the serum ST2 levels were positively correlated with endoscopic activity for both UC and CD patients (Figure 5).[117] In another study, Díaz-Jiménez, D. et al. evaluated the changes in ST2 levels in response to treatment with anti-inflammatory drugs or immunomodulators in UC patients, with the use of ELISA testing.[118] 18 patients positively responded to the treatments given while 6 patients showed no response. Results showed that the median ST2

concentration in positive responders changed from a baseline of 174 pg/ml, down to 87 pg/ml. ST2 levels in non-responders increased from 336 pg/ml to 385 pg/ml during the cases of reactivation episodes for UC. The results show a positive correlation between the levels of ST2 in patients with UC that responded to treatment. The results of these studies indicate that ST2 serum is a useful biomarker to measure the clinical course of IBD patients.

7. TNFAIP6.: Tumor necrosis factor alpha-induced protein 6 (TNFAIP6), also known as TSG-6, is a protein that is secreted during times of inflammation in various cell types.[119] It is a 35 kDa glycoprotein with an N-terminal sequence that shows homology to the Link module, a conservative sequence in hyaluronan (HA)-binding proteins; as well as a C-terminal half that shares sequence homology to the CUB domain, a chain of complement component C1s/C1r, uEGF, and BMP-1, which is a protein involved in the development of sea urchin embryos.[119,120] Due to these specific homologies it has been suggested that TNFAIP6 plays a vital role in extracellular matrix formation or developmental properties.[120] While this protein is rarely found in unstimulated cells or tissues, TNFAIP6 is rapidly unregulated in the presence of pro-inflammatory cytokines TNF and IL-1.[119] TNFAIP6 has been shown to reduce neutrophil infiltration in cells during acute inflammation, as well as reduce levels of inflammatory modulators. This anti-inflammatory effect can be attributed to the proteins Link module.[119] High levels of TNFAIP6 have been detected in mucosal samples taken from patients with IBD; specifically in mucosal smooth muscle cells.[119] A study by Yu, Q. et al. was the first experiment completed to evaluate TNFAIP6 as potential biomarker to diagnose IBD.[121] Using ELISA, the authors demonstrated that the concentration of serum TNFAIP6 in UC, CD patients and healthy controls were 5.8 ng/ml, 5.4 ng/ml, and 2.4 ng/ml, respectively. This study revealed that the levels of TNFAIP6 were significantly correlated with other inflammatory markers of IBD, such as ESR and CRP, as well as serum TNF-α. The serum levels for TNFAIP6 and CRP were tested against the disease activity in IBD using the CD Activity Index in CD patients, and Mayo Score in UC patients. Results showed that the correlation coefficients (r) were comparable for the CD activity index with values of 0.378 and 0.39 for TNFAIP6 and CRP, respectively; and for the UC Mayo Score, TNFAIP6 serum had a higher correlation value than that of CRP with values of 0.65 and 0.51, respectively.[121] While CRP shows higher accuracy for measurement, TNFAIP6 still proves useful for the monitoring of IBD disease activity and should be considered in further studies as a potential biomarker for diagnosing IBD.

B. Fecal biomarkers.

1. Calprotectin.: Calprotectin is a calcium-binding protein that was first isolated from granulocytes back in 1980. It is mainly found in the cytosol of neutrophils, but can also be detected in monocytes and reactive macrophages in lower quantities.[122] Calprotectin possesses antimicrobial and antiproliferative properties and belongs to the S100 family of proteins, which are responsive to acute and chronic inflammation, as well as various malignancies.[123] Calprotectin is a heterodimeric protein composed of subunits S100A8 and S100A9; previously known as MRP8 and MRP14, respectively, for their abundant presence in myeloid cells.[124] These subunits are low molecular weight proteins each comprised of two calcium binding EF-hand domains, otherwise known as the helix-loop-helix formation. Each monomer has four α-helices, and two loops where a calcium ion can bind. In the

presence of calcium, the calprotectin heterodimeric complex can undergo conformational changes, resulting in a heterotetrametric formation (Figure 6).[125] Additionally, these conformational changes expose hydrophobic areas on the S100A8 and S100A9 monomers, which allows for the binding of different target proteins and improves the affinity for transition metals, such as zinc.[124]

During inflammation, calprotectin can be detected in elevated levels in blood plasma, cerebrospinal fluid, synovial fluid, urine, and feces.[124] The concentration levels of calprotectin present in the feces of subjects is ca. six times greater than those found in plasma.[126] The calprotectin concentration in healthy subjects is found to be around ~ 34 μg/g in feces. For patients with CD, the concentration increases significantly to ~ 3200 μg/g; and for UC patients it is around ~1900 μg/g.[127] Due to the large difference in concentration of calprotectin between healthy patients and those with CD or UC, several studies have used fecal calprotectin as an indicator for inflammation in patients.[123,125,126,128] This protein is resistant to bacterial degradation and remains stable for up to 1 week in fecal samples at room temperature. Since fecal sampling is non-invasive, detection of calprotectin is an extremely valuable method for collection, storing and correlating concentrations to inflammation.[123,127] A meta-analysis performed by Freeman, K. et al. examined the use of fecal calprotectin to diagnose IBD, through various methods of measurement: ELISA, fluorescence enzyme immunoassay (FEIA), and Quantum-Blue point-of-care test.[129] Sensitivity from different assays ranged from 85% to 94%, and the specificity ranged from 67% to 88%. Calprotectin concentration can also be used to measure effect of interventions. Sipponen, T. et al. studied the effect of anti-TNF-α treatment on calprotectin levels and found that the median fecal concentration in CD patients decreased from 1200 μg/g to 130 μg/g through treatment (Figure 7).[130]

Calprotectin testing is positively correlated with the endoscopic disease activity of IBD.[131] A recent study showed fecal calprotectin to have a sensitivity of 88% and a specificity of 100% in UC patients ($P < 0.0001$), and a sensitivity of 100% and specificity of 67% in CD patients ($P < 0.001$). Fecal calprotectin testing has been approved by the Food and Drug Administration (FDA) and is recommended by gastroenterological societies for its usefulness in the diagnosis and monitoring inflammation in IBD patients.[129] Due to the successful nature of this biomarker for IBD, Bühlmann of Switzerland developed an at-home testing kit for the rapid detection of fecal calprotectin, called IB*Doc*.[132] This test allows patients with IBD to measure fecal calprotectin levels at home with the use of a smartphone. A study completed by Røer, J. M. et al. examined the usability of this rapid test in a clinical setting.[132] 59 IBD patients were enrolled in this study. Results obtained from IB*Doc* positively correlated with the ELISA results ($P < 0.001$), confirming the successful use of the self-testing system. Similar results were obtained from other studies.[133,134] Taken together, fecal calprotectin is a very useful biomarker for the diagnosis and monitoring of inflammation in IBD patients.

2. S100A12.: S100A12, also known as Calgranulin C, is a calcium-binding pro-inflammatory protein that belongs to the S100 protein family.[135] This family of proteins is known for their consistent structure, made of two calcium-binding sites present on EF-hand domains, and are named for their ability to be 100% soluble in ammonium sulfate.[135,136]

S100A12 is expressed almost exclusively in activated neutrophil granulocytes; and targets the protein RAGE, the receptor for advanced glycation end products, which is expressed on endothelial cells, mononuclear phagocytes, and lymphocytes.[136] Calcium ions are required for S100A12 to bind RAGE and undergo a conformational change. This change in formation results in a concave hydrophobic surface in the C-terminal domain, which becomes the target recognition site for the protein RAGE.[135] Each EF-hand domain, consisting of the helix-loop-helix formation, can bind one calcium molecule; thus, each monomer of the protein can bind two calcium molecules and one target protein. The calcium-binding sites, designated L1 and L2, can be seen in Figure 8 represented by the yellow spherical Ca^{2+} molecules that fall between helix I and helix II (pink), and helix III and helix IV (teal), respectively. Studies have demonstrated that S100A12 undergoes a cellular translocation when interacting with calcium ions.[137] While this protein is normally located in the cytosol of neutrophils, it travels to both the membrane and cytoskeletal compartments when bound to calcium. S100A12 binds to cell surface receptor RAGE on inflammatory cells, which results in the activation of several intracellular signal cascades.[138] This ligation specifically activates nuclear factor (NF)-κB transduction pathway and MAP-kinase pathway—leading to the production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-1b.[136,139] Several reports elaborate S100A12 and its role in various inflammatory diseases, these include rheumatoid and psoriatic arthritis[138], cystic fibrosis[140], various respiratory disorders[141], and IBD.[135] A study performed by Foell, D. et al. determined the tissue expression of S100A12 in IBD patients.[142] The results of the study show that concentration levels for S100A12 were around 470, 400 and 75 ng/ml in patients with active CD, active UC, and healthy controls, respectively.[142] A more recent study demonstrated that the levels of S100A12 secreted in the feces of children with IBD was significantly higher compared with healthy controls.[143] ELISA revealed that the median fecal concentration of S100A12 was ~ 95 μg/g (6 – 350 μg/g) in IBD patients, compared to ~ 0.7 μg/g (0.4 – 18 μg/g) in healthy controls. With a cutoff value of 10 μg/g fecal S100A12, the sensitivity and specificity to diagnose IBD from healthy controls were 96% and 92% respectively. Since S100A12 has been positively identified in the feces of IBD patients, this biomarker could potentially be used as an effective non-invasive self-testing method.

3. Lactoferrin.: Lactoferrin (LTF) is an iron-binding glycoprotein whose function is to transport iron in blood serum. LTF also plays an important role in immune defense.[144] This protein can be found in most mammalian exocrine secretions, such as tears, saliva, gastrointestinal fluids, or breastmilk, and in the secondary granules of neutrophils. LTF is a monomeric 80 kDa polypeptide chain comprised of 692 amino acids, and organized into two homologous structured lobes, designated *N*- and *C*-lobe, that binds a single ferric ion (Figure 9).[145] The two lobes are connected by a hinge region, containing part of an α-helix, which provides the molecule with added flexibility.[144] In addition to binding Fe^{3+} ions, LTF has the ability to bind copper, zinc, or manganese ions for different biological processes.[146] LTF is involved in numerous activities related to host protective effects and regulation of homeostasis in mammals that apply to the immune system, including cancer.[147] It is a primary innate immune component during acute septic inflammation, and is released in elevated levels from neutrophils as a strong antimicrobial agent against a broad spectrum of bacteria, fungi, yeast, viruses, or parasites.[144,145]

LTF levels are shown to increase significantly in the biological fluids of patients who suffer from various inflammatory diseases.[147] Inflammatory processes begin with the activation of sentinel cells in response to microbial components or tissue injury, followed by the release of proinflammatory cytokines, including IL-6 or TNF-α. These cytokines activate endothelial cells, which become more permeable to recruit phagocytes from the blood. These neutrophils have large amounts of LTF that are released at inflammation sites. High levels of LTF can inhibit the production of proinflammatory cytokines and increase the number of natural killer cells, as well as induce phagocytosis.[144] LTF has a strong positive correlation to sites of inflammation and is distributed in so many biological fluids, including mucosal and epithelial cells. Kane, S. V. et al. compared the concentration levels of fecal lactoferrin in patients with IBD to healthy controls using ELISA.[148] In healthy subjects, the concentration was found to be around ~1 μg/g, in CD patients it increases to ~440 μg/g, and in UC patients it is ~1100 μg/g.[148] The sensitivity and specificity of fecal LTF to diagnose UC in patients were 81% and 82%, respectively; while the sensitivity and specificity to diagnose CD were 82% and 0.71%, respectively.[74] The effects of intervention on LTF levels in patients using anti-TNF-α therapies demonstrated that LTF could be potentially used to monitor inflammation. LTF concentration levels in CD patients measured using ELISA, decreased from 105 μg/g to 3 μg/g after anti-TNF-α treatment.[130] Fecal LTF testing has been demonstrated to correlate very well with other diagnostic measures. A study by Sipponen, T. et al. demonstrated that fecal lactoferrin correlates better than fecal calprotectin as measured by ileocolonoscopy in CD patients.[149] With a cutoff of 10 μg/g of fecal lactoferrin, the sensitivity and specificity detected were 66% and 92%, respectively. Taken together, LTF fecal concentrations can also be used to monitor inflammation severity in CD patients.

4. Lipocalin-2/NGAL.: Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, is a glycoprotein originally isolated from neutrophils that can be found in monomeric and homodimeric forms, as well as in a NGAL-gelatinase complex.[150] This protein is part of the lipocalin family of proteins, which are small secreted proteins characterized by their ability to bind small hydrophobic molecules and specific cell-surface receptors, as well as their shared macromolecule formation complexes.[151] Lipocalin proteins form a common secondary and tertiary structure called the “lipocalin fold,” which consists of an antiparallel beta barrel structure with eight beta sheets that are held together by hydrogen bonds.[152] NGAL is comprised of 198 amino acid residues; with a 20-residue signal peptide at the N-terminal before the lipocalin domain, and followed by a negatively charged “pit” at the base of the beta-fold barrel [152] (Figure 10). This pit is formed from Aspartate and Glutamate residues, in addition to an unpaired Cysteine residue that is important for binding the gelatinase enzyme matrix metalloprotease-9 (MMP-9), which is responsible for the degradation of matrix components. NGAL has been found to stabilize MMP-9 and often forms this important complex extracellularly, only after both proteins have been secreted.[152,153] The interaction of NGAL with MMP-9 is formed via disulfide linkage, and results in an interference in the degradation of MMP-9 and preservation of enzymatic activity, further promoting the release of growth factors and enhancing tumoral invasiveness via this enzyme.[152,154] While lipocalins have generally been classified as extracellular transport proteins, NGAL is considered a positive acute-phase protein with

anti-inflammatory functions. This iron-sequestrating antimicrobial protein is secreted from activated neutrophils, and can be detected in various biofluids, including blood, urine, and feces.[150] NGAL can be found in low levels in normal adult tissues including but not limited to tissues in the kidney, liver, small intestine, adipose tissue, and bone marrow.[152] Its function in normal tissues is to regulate oxidative stress and provide antimicrobial defenses against bacterial infections.[155] The presence of NGAL is expressed in elevated levels in chronic and acute inflammation and a wide array of benign and malignant diseases effecting various organ including digestive, respiratory, endocrine, and reproductive organs.[152,156] There are many factors that regulate NGAL expression, ranging from pro-inflammatory cytokines, such as interleukins and tumor necrosis factor-α (TNF-α), to various vitamins in the body.[152]

High NGAL expression has been positively identified in IBD patients with inflamed colonic epithelium.[156] In one study with a limited number of patients, i.e. 21 patients with IBD and 23 healthy patients, the median concentration of NGAL present in UC patients was 6 (4 – 15) μg/g, and in CD patients at 5 (2 – 8) μg/g in contrast to healthy subjects expressed lower concentrations at around 0.3 (0.1 – 0.4) μg/g.[157] The sensitivity and specificity to diagnose active IBD from the subjects were 94.7% and 95.7%, respectively. Additionally, the use of NGAL to diagnose IBD was evaluated in correlation with the Endoscopic Index of Severity (CDEIS).[158] NGAL levels were detected by ELISA and showed the sensitivity and specificity to be 85.7% and 45.6% respectively. Based on these studies, NGAL is also considered as a potential biomarker for IBD.

5. Myeloperoxidase (MPO).: MPO is an enzyme found in the azurophil granules of neutrophils, and is used as a microbicidal agent that can attack foreign materials.[159] This strongly cationic and glycosylated 144 kDa protein has the ability to form a wide variety of oxidants.[160] It belongs to the mammalian peroxidase superfamily. It is a hydrogen peroxide oxidoreductase and produces cytotoxic acids, such as hypochlorous acid. The reactive oxidants created through this reaction have the ability to kill bacteria, fungi, metazoan organisms, and viral pathogens to protect the body; however, they also can damage host tissue as a side effect.[161] MPO is comprised of two identical dimers that are linked via disulfide bridge; each dimer has one light subunit, and one heavy subunit that contains a protoporphyrin IX group with a central iron ion (Figure 11).[162] These hemes are joined to the apoprotein through two ester linkages and one sulfonium ion linkage (Figure 11). The three linkages attaching the heme to the protein is unique compared to other heme proteins—to accommodate varying binding sites for the protein. Due to the strong cationic conditions of MPO, it can bind readily to the negatively charged endothelial membranes of cells.[160] MPO regulates multiple aspects of inflammatory responses through its interaction with hydrogen peroxide. This interaction results in the inactivation of secreted granule contents and influences other functions of stimulated neutrophil granules.

The MPO concentration is directly proportional to the number of neutrophils.[163] Since neutrophils play a vital role in the inflammatory processes of IBD and are directly correlated to MPO, studies to correlate inflammation to MPO levels were undertaken.[164] It was demonstrated that median concentration of MPO was ca. 4, 100 and 60 μg/g in healthy, UC and CD patients, respectively. These results show a significant difference in the MPO

levels of IBD patients compared to healthy subjects, which indicates the potential of MPO as a biomarker for IBD. MPO is also closely correlated to other biomarkers that are present during intestinal inflammation, such as lactoferrin and calprotectin.[165] Masoodi, I. et al. used ELISA to evaluate the concentrations of these biomarkers compared to healthy controls. The results showed MPO to have a better sensitivity than CRP when diagnosing UC in patients (89% versus 24%); however, the specificity was lower (51% versus 100%).[166]

6. Matrix metalloproteinases (MMPs).: MMPs are a group of zinc-dependent endopeptidases with a conserved catalytic domain, and a Zn^{2+} present at the active site; collectively termed matrixins.[167,168] (Figure 12). Their basic mechanism of action, the degradation of extracellular matrix (ECM) and specific proteins, is used to regulate immune responses and various cellular behaviors. The degradation of extracellular proteins by MMPs is essential for any cell to properly interact with its surroundings, and allows for multicellular organisms to develop and function normally.[169] Furthermore, MMPs play major roles in diverse physiologic and pathological processes, including but not limited to: embryonic development, tissue morphogenesis, wound repair, inflammatory diseases, and cancer. Due to their importance in many biological processes MMPs are tightly regulated at transcriptional and post transcriptional levels; as well as at a protein level during activation, interaction with ECM components, inhibition, and cell surface localization.[168,169] While MMPs are not typically expressed in normal healthy tissues, they can be detected in all repair processes, diseases or inflamed tissues, and in all cells that are grown in culture.[170] Historically, MMPs have been classified into divisions by their substrate pcollagenases, gelatinases, stromelysins, and matrilysins.[167] More recently MMPs are grouped according to structure, with eight distinct groups of structural classes; five of which are secreted, while the other three are membrane-type MMPs. Secreted MMPs include minimal-domain, simple hemopexin-domain-containing, gelatin-binding, furin-activated secreted, and vitronectin-like insert MMPs. While membrane-type MMPs include: transmembrane, GPI-anchored, and type II transmembrane MMPs.[167] All MMPs possess a distinguishable and highly conserved zinc-binding motif, HEBXHXBGBXHZ.[169] In this motif, H are histidine residues, E is glutamate, G is glycine, B represents a bulky hydrophobic residue, X can be variable, and Z is a family-specific residue. Additionally, all MMPs have an N-terminal signal sequence, that is removed once the protein is directed to the endoplasmic reticulum. Furthermore, the cysteine switch motif PRCGXPD in the propeptide is present to keep MMPs in their zymogen form (proMMP).[168] MMP-9 specifically, along with MMP-1, 2, and 3, have been detected in significantly high levels in colonic biopsies from IBD patients.[171] MMP-9, also known as gelatinase B, has three repeat domains of type II fibronectin domain into the catalytic domain, which is used for binding to gelatin, collagens, or laminin.

Baugh, M. D. et al. explored the expression of multiple MMP-1, MMP-2, MMP-3, and MMP-9 in patients with IBD.[172] The intestinal mucosa of patients with UC, patients with CD, and healthy controls were sampled and tested for these MMPs using zymography image analysis and western blot testing. Furthermore, in patients IBD tissue biopsies were taken from inflamed intestinal mucosa as well as non-inflamed mucosa (Figure 13).[172,173] Results revealed that the most MMP concentrations were in the samples of inflamed mucosa,

in comparison to healthy controls and non-inflamed mucosa. The MMP levels in the non-inflamed mucosa samples were higher than healthy controls. Additional studies have examined the expression of MMPs in the intestinal mucosa of IBD patients and their role in the disease and in animals. The results demonstrated that the major sources of these MMPs were infiltrating macrophages.[174,175] A recent study evaluated the diagnostic value of fecal MMP-9 in different types of IBD using ELISA.[171] The results showed that the median concentration of fecal MMP-9 was 1.5 ng/ml in active CD patients, compared with 0.6 ng/ml in inactive CD patients; and 6.2 ng/ml in active UC patients, compared to 0.7 ng/ml in inactive UC patients. Fecal MMP-9 did not show a strong correlation with any of the disease activity indicative of CD. However, it did show a strong association with the clinical and endoscopic activities of UC. For UC patients with positive endoscopic activity, the sensitivity and specificity of fecal MMP-9 to diagnose UC were 96% and 75%, respectively.

7. Intestinal alkaline phosphatase (IAP).: IAP belongs to a superfamily of metalloenzymes and is considered a crucial mucosal defense factor that works to maintain gut homeostasis.[176] While this enzyme is rarely found in the stool of healthy subjects, it is present in the apical microvilli of the brush border of enterocytes found in the intestine. It is secreted apically and basolaterally by the enterocytes, and is expressed in the intestinal lumen, as well as the bloodstream, in response to inflammation.[176,177] IAP has four major functions: regulation of bicarbonate and duodenal surface pH, long chain fatty acid absorption, detoxification of pathogen-associated intestinal inflammation, and regulation of the gut microbiome.[178] These important functions of IAP are necessary for the maintenance of homeostasis in the gut microbiome, and have systemic anti-inflammatory effects.[176] The structure for IAP is a dimeric glycoprotein that is comprised of two identical monomers, with two buried cysteine residues.[179] IAP is 90 – 98% homologous with the other two tissue-specific AP isozymes: placental AP and germ cell AP. Figure 14 showed the structure of AP monomer.[180] It is a zinc-containing enzyme, and the full structure of IAP can be compared to that of the E. coli enzyme—as it is the most well studied. Perhaps one of the most important roles that IAP plays in protecting the gut microbiome is the ability to detoxify the endotoxin lipopolysaccharide (LPS), which is a major component of the outer membrane of gram-negative bacteria that composes most of the mammalian intestinal bacteria in the microbiome.[176] Activation of LPS leads to a signaling cascade that releases pro-inflammatory cytokines. The ability of IAP to counteract these processes in the gut microbiome has brought this potential biomarker to the forefront of IBD studies. A study by Molnár, K. et al. tested the IAP concentrations present in the intestinal mucosa of IBD patients, in comparison to healthy subjects.[181] In addition, the samples obtained from UC patients were taken from inflamed and non-inflamed areas, for additional comparison of IAP levels. The results revealed that IAP concentrations in the inflamed mucosa of patients with CD and UC were lower than healthy subjects by 22% and 20%, respectively. When comparing the mucosa samples from inflamed and non-inflamed areas in UC patients the IAP levels in the non-inflamed area were normal, with inflamed areas having much lower levels of IAP. Another study performed by Park, Y. S. et al. evaluated the expression of IAP in the disease course of CD patients.[182] 32 CD patients participated in the study and were monitored over the course of 14 months after biopsy sampling. ELISA tests detected IAP concentrations in the intestinal mucosa; and samples were taken from inflamed and

non-inflamed areas of the colon. Results showed that 31.3% of patients had lower IAP expression in inflamed mucosa compared to non-inflamed mucosa; the median value of IAP in inflamed mucosa was ~ 43 ng/ml, compared to 26 ng/ml in non-inflamed mucosa. Of the patients with this disparity in IAP levels, 90% experienced clinical recurrences of CD. This suggests that the concentration of IAP in the intestinal mucosa of CD patients may be associated with clinical recurrences of the disease, proving its usefulness as a potential biomarker for monitoring IBD.

miRNA:

miRNAs are a group of small non-coding RNAs, approximately 18–22 nucleotides,[183] that act as regulators for post-transcriptional gene expression and are found across species. Their discovery was first described in 1993 in *Caenorhabditis elegans.*[184] miRNA expression is essential in various human diseases such as cancer, autoimmune, cardiovascular, and neurodegenerative diseases.[185–187] The miRNAs are circulate in the human peripheral blood in a stable form, and also present in other body fluids such as urine, saliva, milk, cerebrospinal fluid, and feces.[188–191] The miRNAs are engaged in disease origin and development, and some are pathology-specific.[192] Thus, changes in miRNA expression profiles have been addressed for applications in early detection, prognostics and diagnostic classification. Most recent research in the IBD field has measured circulating miRNAs in body fluids such as blood or feces and in homogenized tissue biopsies using microarray profiling techniques RT-qPCR next generation sequencing (NGS).[193–197] Even though many miRNAs are reported, we mainly focused on the miRNAs found in peripheral blood and feces samples and are listed in Table 4. MiR-21 and miR-155 have repeatedly been identified and seem to be the most studied miRNAs related to IBD.[198–200] MiR-21 is possibly the most intriguing miRNA involved in IBD, with associations between miR-21 and IBD replicated in several studies and functional relevance reported in mouse models of IBD.[201] In a recent research, Peng Chen et al. reported[202] serum microRNA146b2-5p (miR2-146b2-5p) expression was 2.872- and 2.722- fold higher in patients with CD and UC, respectively, than in healthy control ($P = 0.0043$). Another study found serum samples from IBD patients showed a higher level of miR-16, miR-21, and miR-223 than controls and was higher in CD than in UC patients.[203] More significant miRNA expression changes were observed in feces from IBD patients for all studied miRNAs with the highest expression of miR-155 and miR-223 in testing and validation cohorts. The miR-21, miR-155, and miR-223 display significant levels and could potential be considered as biomarkers for IBD.

Conclusion:

The main diagnostic procedures used to establish an accurate diagnosis for IBD patients are currently the combination of endoscopy with multiple biopsies. These procedures are not user-friendly as they are invasive and uncomfortable for most patients. Since individuals of all ages, including young children, have the potential to develop IBD, non-invasive, user-friendly methods are needed to diagnose IBD, monitor inflammation and determine therapy effectiveness. Several diagnostic methods and biomarkers have been examined for the use of diagnosing IBD and some have demonstrated to possess considerable promise in the diagnosis of IBD. As the intricate mechanisms governing the inflammatory pathways are

uncovered, more specific, sensitive biomarkers will emerge. In addition to these biomarkers, genetic variations in patients were studied to evaluate the differences between UC and CD..[204] CD was found to be associated with minor *NOD2*, *TLR4* 299Gly, *TNF-α* G-308A, *IL-6* G-174C, and *IL-1RN* VNTR A2 variants, while UC was associated with *IL-1RN* VNTR A2 variants. Genetic studies can assist in the diagnosis of IBD and differentiate between CD and UC. MicroRNAs (miRNAs) are also found play an important role in IBD. MiRNAs are short, non-coding, single-stranded RNAs, which affect the gene expression by playing as polymorphisms in miRNA binding site in IBD.[205,206]

Among all the biomarkers, fecal biomarkers (Figure 15) are the most non-invasive and have the added ability to be used at home when monitoring IBD. The ideal biomarkers should have these features, it must be highly sensitive for detecting inflammation in the gastrointestinal tract, concentration should reflect the severity of inflammation and responsive to the change of inflammation after treatment and the biomarker should be temperature stable.[207] Additional criteria include sample volume, ease of collection and good correlation between analytical LOD and clinical sensitivity. We envision a diagnostic that uses multiple biomarkers in a device to identify and monitor disease progression leads to improved positive and negative predictive values. We note that the interplay between diet, gut microbiota and the immune system are important factors to consider when developing these biomarkers as diagnostics. These biomarkers must be benchmarked against established invasive methods in an objective manner before they can be considered for clinical applications. Although a lot of non-invasive biomarkers have been effective in monitoring IBD, large scale clinical trials and further development as a point-of-care (POC) diagnostic must be completed. If further research on these biomarkers proves that it is difficult to accurately identify colitis, these biomarkers could still have considerable value in monitoring disease progression after a patient has been conclusively identified as suffering from IBD. At the current time, IBD patients must visit their physicians regularly for invasive colonoscopies. We envision a user-friendly POC diagnostic that meets the ASSURED (affordable, sensitive, selective, user-friendly, rapid, and robust, equipment-free, deliverable) criteria [208]. Patients could use in the privacy of their homes and upload the results to their electronic medical records. With the acceleration of telehealth practices, physicians could adjust the medication dose or suggest alternative therapies, thereby reducing the number of hospital visits and invasive procedures. Since IBD is life-long disease, the development of POC diagnostics will allow patients to monitor their status of severity and drug management at home without invasive procedures. The need for POC diagnostics is evident, and the further research into biomarkers that are increased or decreased or in IBD patients, their correlation to inflammation and their application in POC diagnostics will be essential to decrease burden on IBD patients.

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Author bio-sketches:

Suri S. Iyer: Dr. Suri S. Iyer is a Professor at Georgia State University. The long-term goals of the Iyer group are to develop inexpensive, user-friendly devices that could be used at primary care physician's offices, low resource settings and for self-testing in the privacy of homes. To this end, his group has been developing glycan-based diagnostics for the capture and detection of toxins and pathogens such as Shiga toxin, influenza virus, norovirus and malaria and HIV. Most recently, he is collaborating with Dr. Merlin to develop POC (Point Of Care) diagnostics to monitor inflammation in IBD patients.

Didier Merlin: Didier Merlin is a Professor at Georgia State University and Research Career Scientist at Veterans Affairs Medical Center, Decatur, GA. His research area is the study of intestinal epithelia, as directly related to IBD. Among the research areas, Dr. Merlin's research group is focused on the role of PepT1 in intestinal inflammation, understanding the propagation of the intestinal inflammation that is mainly characterized by a loss of epithelial cell/cell interactions that result to a loss of the intestinal fence or barrier function and drug delivery to reduce inflammation in IBD patients. Most recently, his group is collaborating with Dr. Iyer's group to develop POC diagnostics to monitor inflammation in IBD patients.

Dandan Liu: Ms. Dandan Liu is a graduate student at Georgia State University. Her research focus is on the development of assays to detect biological relevant analytes. Ms. Liu has been developing assays for three diseases; detection and differentiation of influenza viruses and *S. pneumoniae*, detection of norovirus and detection of inflammatory biomarkers that are elevated in IBD patients.

Dr. Varma Saikam: Dr. Saikam is a postdoctoral fellow at Georgia State University. His research is on the development of assays to detect pathogens and clinically relevant analytes. Dr. Saikam has been developing point of care diagnostics for a number of diseases including IBD.

Katie A. Skrada: Ms. Katie A. Skrada is a graduate student in Georgia State University. Her major is biochemistry. She is involved in the developing assays that could be potentially used in POC diagnostics to monitor inflammation in IBD patients.

Data availability statement:

No experimental data was generated for this review article

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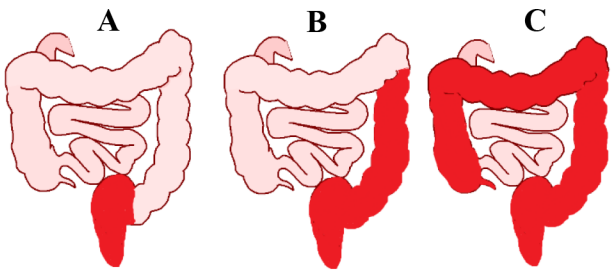

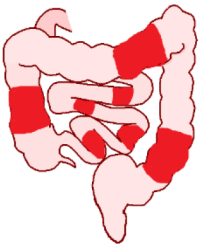

UC

- Affects only the colon.
- Continuous inflammation pattern, affects inner epithelial layer of colon
- Symptoms: fatigue, fever, rectal bleeding, tenesmus, urgency proctitis, diarrhea, abdominal cramping, constitutional symptoms

CD

- Can affect any part of gastrointestinal tract
- Un-continuous, patchy inflammation, can affect any layer of bowel wall
- Symptoms: abdominal pain, fever, clinical signs of bowel obstruction or diarrhea with passage of blood or mucus

Figure 1. Three different types of UC.

A. Proctitis; B. Left-side colitis; C. Extensive colitis. And Differences between UC and CD[9]

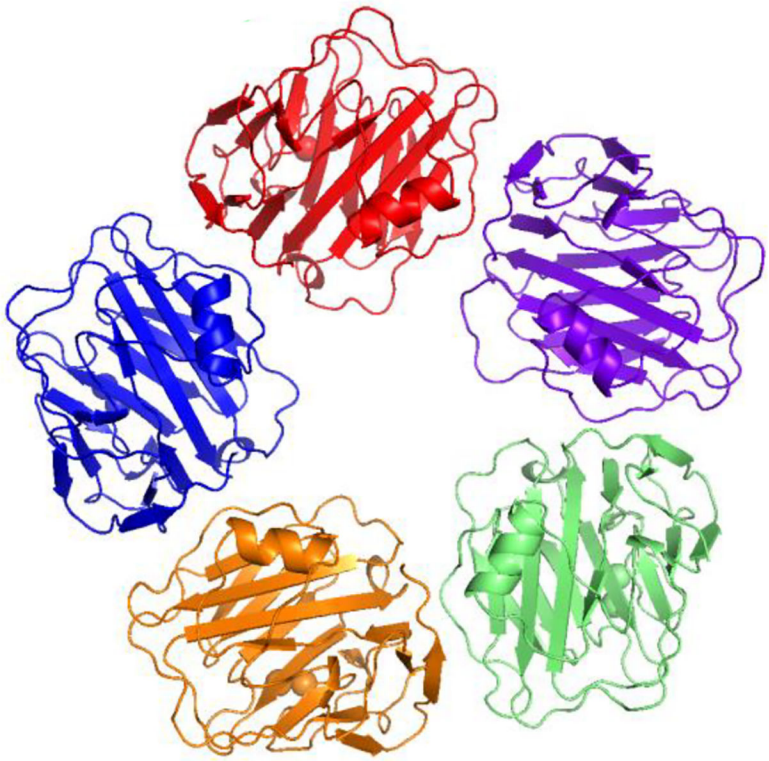

Figure 2. Molecular structure of CRP.

The structure was downloaded from the NIH PDB database. Molecular structure for CRP (PDB: 1GNH)[62] consists of five identical polypeptide subunits, each represented by a different color.[230]

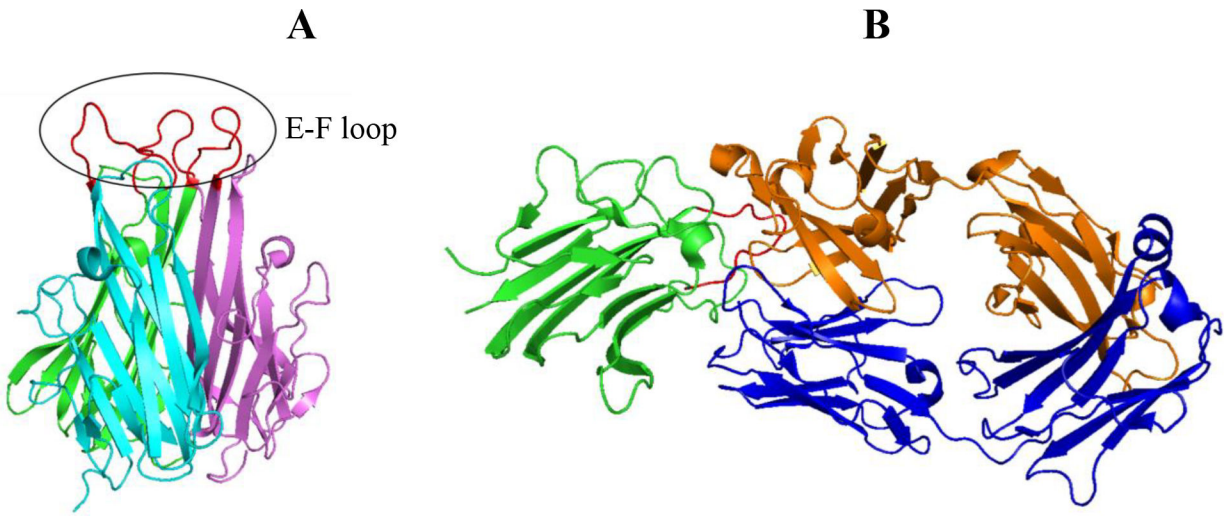

Figure 3. Overall structure of human TNF-α and its monomer binding with infliximab.
Structures downloaded from NIH PDB database. A. Overall structure of TNF-α (PDB: 1NTF).[222] It is a trimer, each monomer shown in different colors (green, cyan, purple). The E-F loop is shown in red, which plays a central role in antibody-antigen binding. B. TNF-α monomer binding with infliximab Fab (PDB: 4G3Y).[223] Infliximab Fab heavy chain is shown in orange, while the light chain is shown in dark blue. The TNF-α monomer is shown in green and the E-F loop is shown in red. One trimer of TNF-α can bind at most three infliximab molecules.[223]

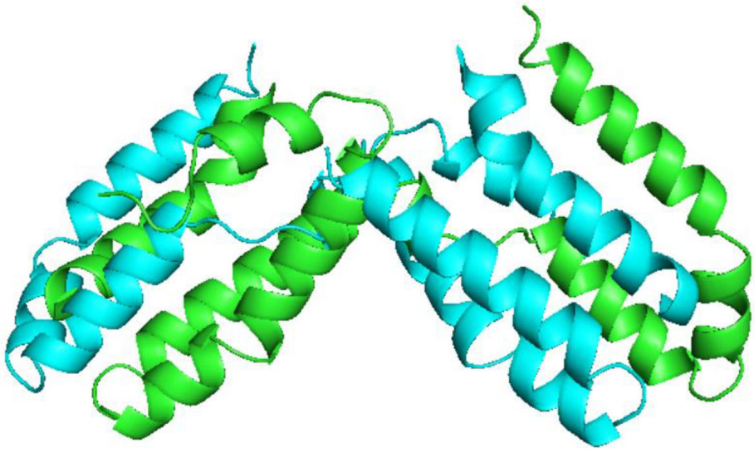

Figure 4. Overall structure of IL-10 dimer.

Structures downloaded from NIH PDB database (PBD: 1INR).[224] Each monomer is shown in green and cyan color respectively. One monomer consists of six helices.[231]

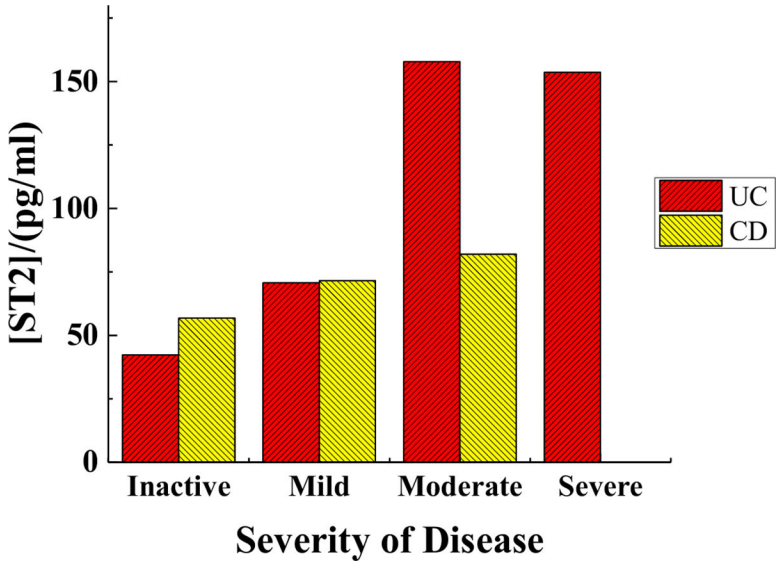

Figure 5. ST2 serum concentration levels in correlation to endoscopic disease activity in patients with UC and CD.

[117] The ST2 serum concentrations were measured in patients with UC and CD, with varying levels of severity for each disease. The endoscopic disease activity was measured using CCAI for UC patients, and CDAI for CD patients. No patients with CD fell into the category of severe disease activity based on CDAI.

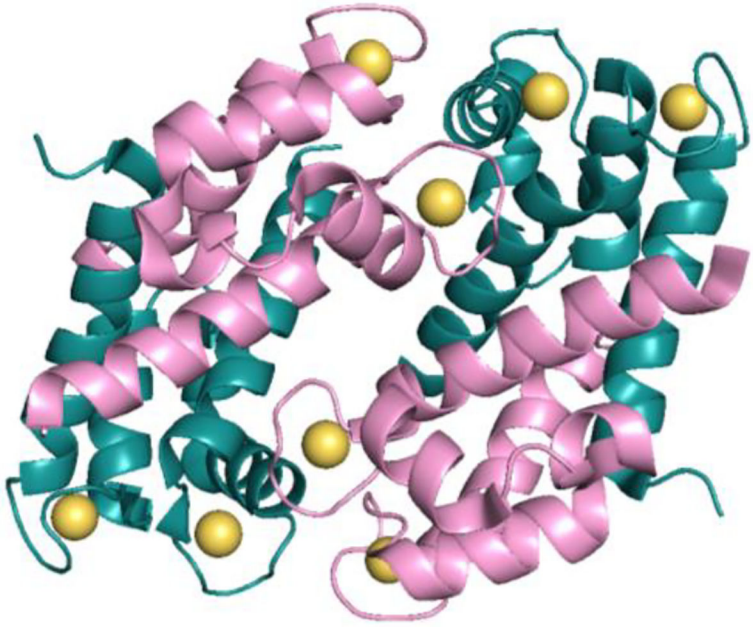

Figure 6. Overall molecular structure of calprotectin heterotetramer.

The structure downloaded from the NIH PDB database. The heterotetramer structure of calprotectin (PDB: 1XK4)[125] is composed of two heterodimer protein subunits: S100A8 and S100A9, forming the total formation structure $(S100A8/S100A9)_2$. S100A8 subunit is shown in pink color, S100A9 is shown in teal color. Calcium ions bound to each EF-hand formation are shown as yellow spheres.

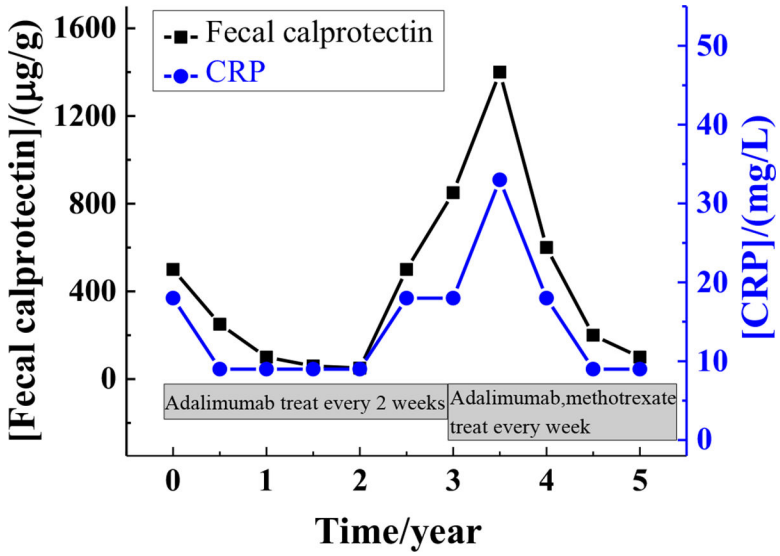

Figure 7. The changes in fecal calprotectin and CPR concentrations for IBD patients in response to treatment.

[225] After receiving adalimumab treatment, the concentration levels of fecal calprotectin and CRP show a significant decrease. As patients develop resistance to the treatment, the concentration levels of these biomarkers begin to increase again. Altering the medications given to include methotrexate, in addition to giving these treatments more frequently, result in decreasing levels of biomarker concentrations.

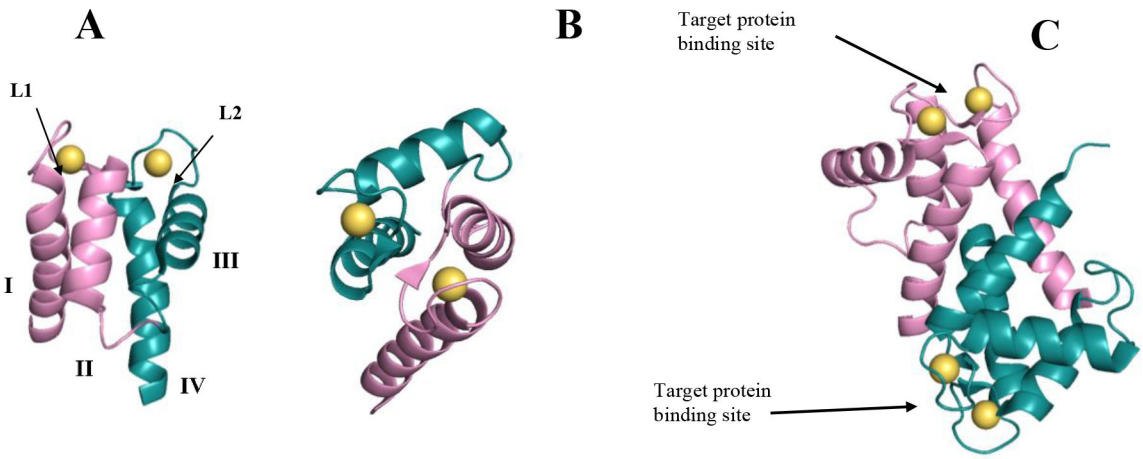

Figure 8. Molecular images for a single unit of protein S100A12.

A: Side view; B: Top view. Structure downloaded from the NIH PDB database. S100A12 (PDB: 1E8A)[226] consists of two EF-hand domains, which are shown as teal color and pink color. The EF-hand domain is a helix-loop-helix formation, consisting of a total of four α-helices which are labeled I, II, III, and IV in image A. These helices can be seen clearly in both image A and B, from different angles. Within each EF domain is a calcium binding site, labeled L1 and L2 in image A, and Ca^{2+} are shown as yellow sphere. C: Represents the homodimer formation of S100A12, which is comprised of two molecules aligned in an anti-parallel formation, with a total of eight alpha helices. There are two ligand binding sites in the homodimer, which are labeled with arrows; and are located at the top and bottom of the antiparallel formation to bind two target proteins.

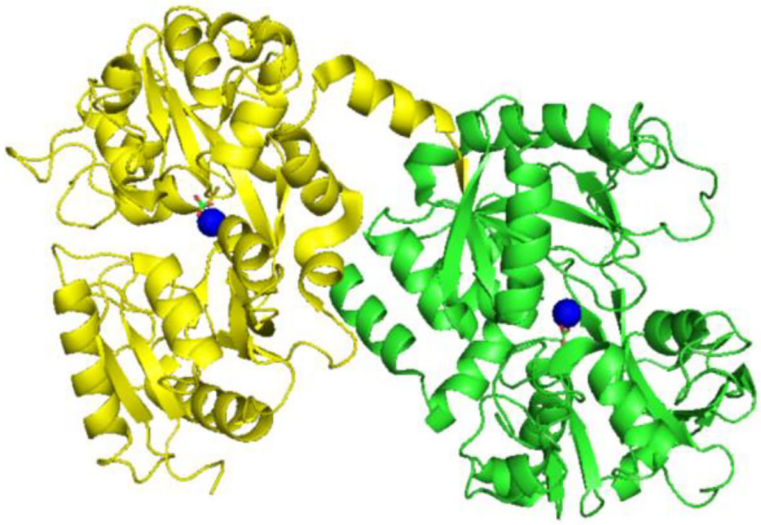

Figure 9. Molecular structure of Lactoferrin (LTF).

Structure downloaded from the NIH PDB database. This structure (PDB: 1SQY)[145] shows the single polypeptide for LTF, color coded according to the structured homologous lobes: *N*-lobe is shown in yellow color and *C*-lobe is shown in green color. In the middle of each lobe is a ferric (Fe^{3+}) ion, represented by a dark blue sphere, along with a carbonate (CO_3) ion, shown as red (O) and green (C) stick figures—which is necessary for Fe^{3+} binding.

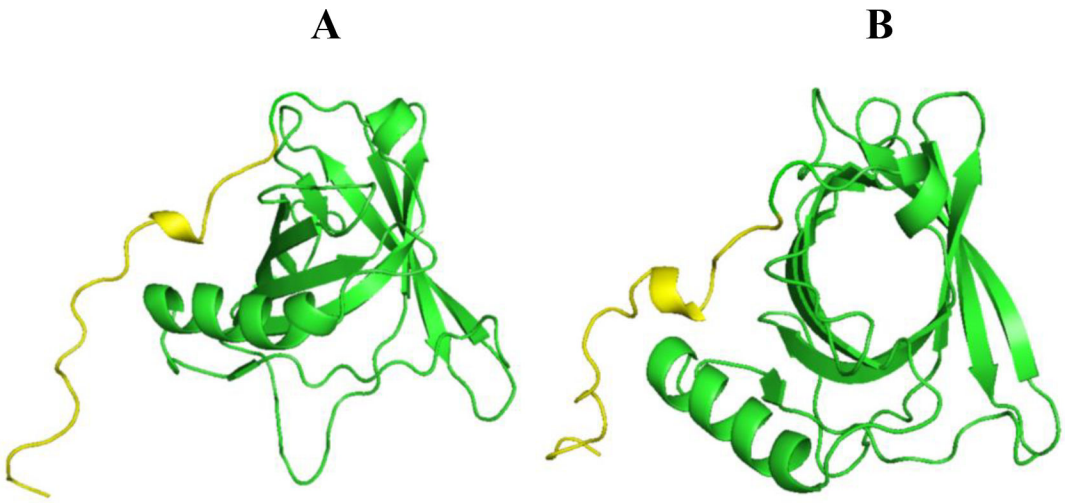

Figure 10. Molecular structure for unbound neutrophil gelatinase-associated lipocalin (NGAL).
A: Side view; B: Top view. Structure downloaded from the NIH PDB database. The molecular structure for a single monomer of NGAL is shown (PDB: 1NGL).[227] The twenty residue-long signal peptide is indicated by the yellow portion; while the rest of the protein, comprised of the “lipocalin fold” antiparallel beta barrel, is shown in green color.

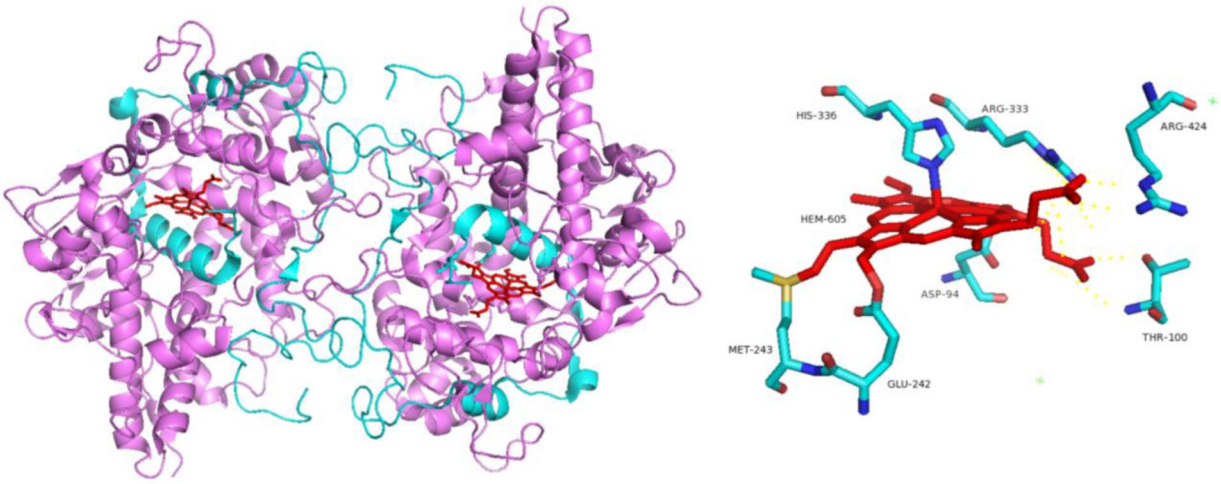

Figure 11. Overall molecular structure for myeloperoxidase (MPO) and interactions between protoporphyrin IX heme.

Structure downloaded from the NIH PDB database. The structure for MPO (PDB: 1CXP)[162] is comprised of two identical dimers. Each dimer has a light chain (shown in blue color) and a heavy chain (shown in purple color). In each heavy chain there is a protoporphyrin IX molecule with a central iron atom, which is shown in red color. The hydrogen bonds are displayed as yellow dashes (right figure). This figure shows the nitrogen atom of His 336 that provides the proximal ligand to the iron of the heme. The methyl groups on the pyrrole ring form ester bonds with the carboxyl group of Glu242 and Asp94, respectively. In addition, the vinyl group on the pyrrole ring forms a covalent bond with the sulfur atom of Met243 and hydrogen bonds with Arg333, Thr100, Arg424 and water molecules.

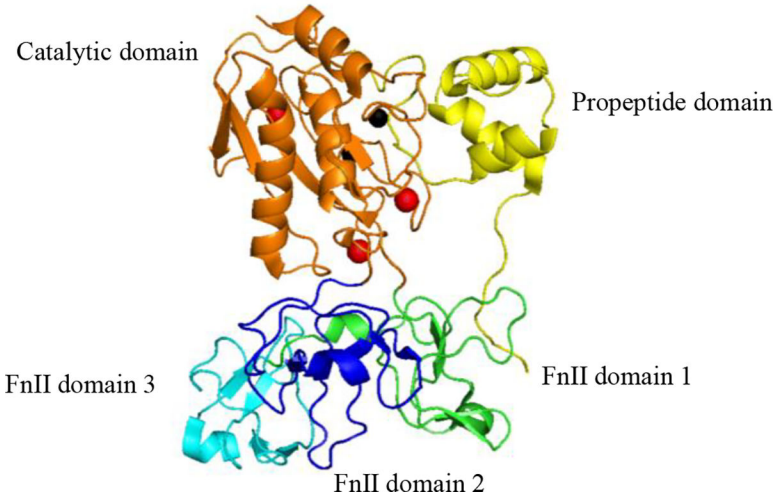

Figure 12. Overall structure of proMMP-9.

Structure downloaded from the NIH PDB database. ProMMP9 (PDB: 1L6J)[228] consists of 5 domains: propeptide (yellow color), catalytic domain (orange color) and 3 Fibronectin type II (FnII) domains (green, blue, and cyan colors). Zn atoms were shown in black color and Ca atoms were shown in red color. ProMMP9 is enzymatic inactive, and it can be activated by cleavage of propeptide. Zn is binding with catalytic domain, which is important for MMP9 enzymatic activity.

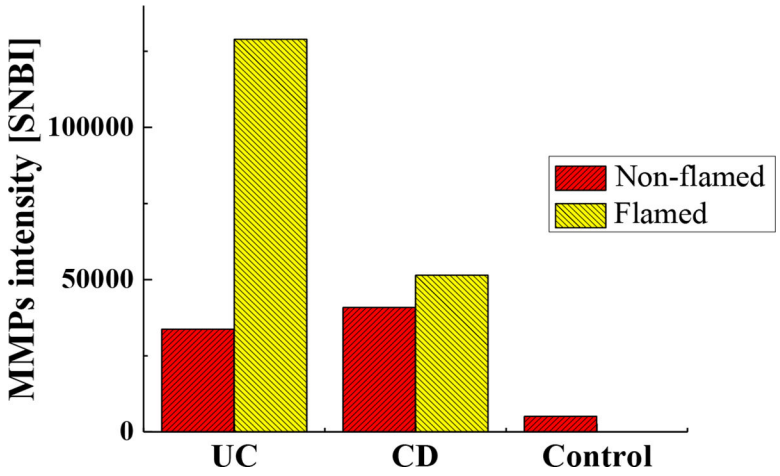

Figure 13. Sum net band intensity levels of MMPs in the intestinal mucosa biopsies from UC patients, CD patients, and healthy controls taken from inflamed and non-inflamed tissues.

[172] Patients with UC and patients with CD were subject to intestinal mucosa samples taken from inflamed areas of colon and non-inflamed areas of the same segment of colon. Healthy controls mucosa tissue was non-inflamed. MMP levels were measured by total proteolysis (sum net band intensity [SNBI]) per lane.

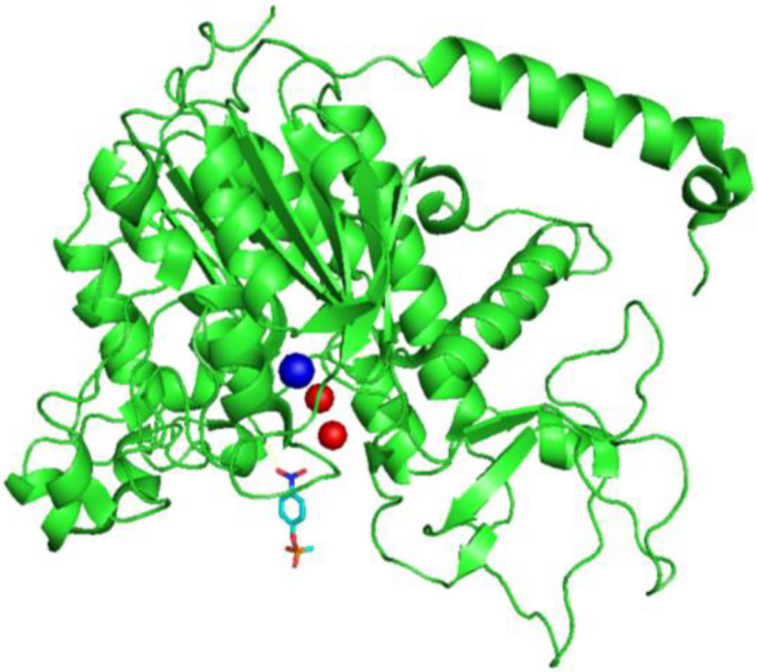

Figure 14. Monomer of human alkaline phosphatase binding with methyl-phosphonic acid mono-(4-nitro-phenyl) ester (PNP).

Structure downloaded from NIH PDB database (PDB: 1ZED).[180] Zinc ions are shown in red sphere, magnesium is shown in blue sphere and PNP molecule is shown in stick. Two zinc ions and one magnesium ion are contained in the active site, and they are crucial for the enzymatic activity. PNP is a substrate analogue, whose phosphate ester bond can be cleaved by alkaline phosphatase.

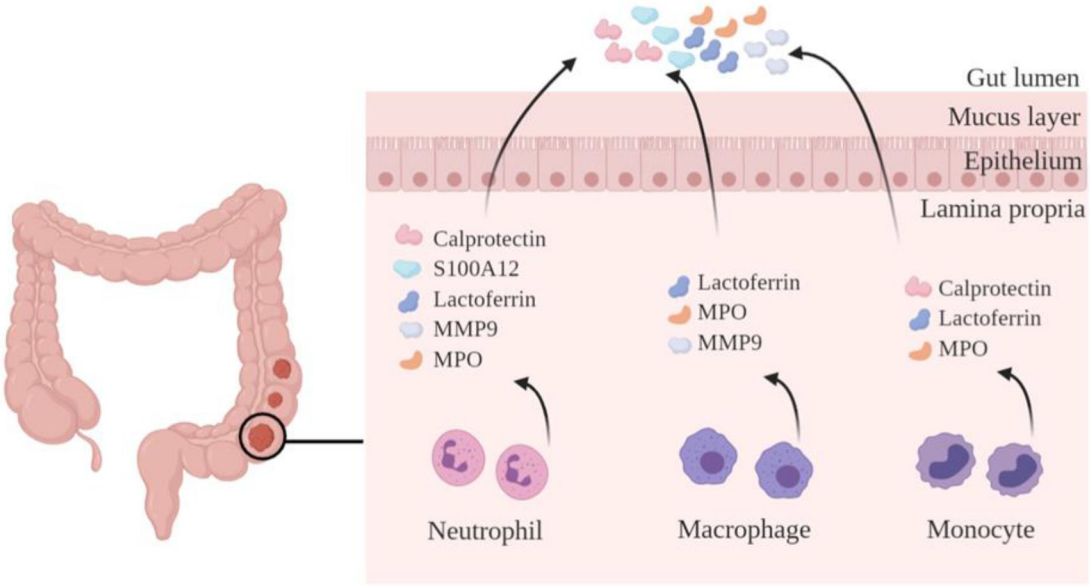

Figure 15. The fecal biomarkers secretion of IBD.

The inflammation in the gut mucosa leads to enhanced migration of innate immune cells, such as monocytes, macrophages and neutrophils to the affected mucosa.[229] These cells secrete inflammatory mediators, such as calprotectin, lactoferrin, MMP9 et al. actively and the mediators are released to gut lumen, which can be detected from feces.

Table 1.

Current treatments for IBD.

Drug		Target	Indication
Anti-inflammatory drugs	5-ASA	PPAR-γ[24–26]	CD, UC
	Corticosteroids	Glucocorticoid receptor[30]	CD, UC
Immunosuppressive agents	6-Mercaptopurine	Hypoxanthine-guanine phosphoribosyl transferase (HGPRT), phosphoribosyl pyrophosphate amido transferase (PRPP amido transferase) et al.[209]	CD, UC
	Azathioprine		CD, UC
	Methotrexate	Dihydrofolate reductase (DHFR)[210]	CD
	Cyclosporine	Calcineurin[211]	UC
Biologics	Infliximab	TNF-α[212]	UC, CD
	Adalimumab		UC, CD
	Certolizumab pegol		CD
	Golimumab		UC
	Natalizumab		CD

Table 2.

Crohn's Disease Activity index variables and weighting factors.

Variable	Weighting factor
Number of liquid or soft stools daily for 7 days.	× 2
Abdominal pain (severity ranges from 0–3) daily for 7 days	× 5
General wellbeing (severity ranges from 0–4) daily for 7 days	× 7
Opiates for diarrhea	× 30
Complications	× 20
Abdominal mass (ranges from 0–5)	× 10
Hematocrit value (men < 0.47, women < 0.42)	× 6
% Body weight from standard	× 1

Table 3.

Biomarkers used for diagnosis of IBD.

Biomarker		Concentration		
		UC	**CD**	**Healthy or remission disease**
Serological biomarker	CRP	20 mg/L	40 mg/L	1 mg/L – 3 mg/L
	ESR *	~30 mm/h		~20 mm/h
	ASCA and p-ANCA			
	NO	15.3 μM	14.5 μM	13.3 μM
	Cytokine (TNF-α, IL-102)	7.6 pg/ml (TNF-α) 144+34 pg/ml (IL-10)	12.7 pg/ml (TNF-α) 132+32 pg/ml (IL-10)	0.02 pg/ml (TNF-α) 44+9.5 pg/ml (IL-10)
	ST2	56.8 pg/ml		30.7 pg/ml
	TNFAIP6	5.8 ng/ml	5.6 ng/ml	2.4 ng/ml
	NGAL[213]	87 ng/ml	90 ng/ml	60 ng/ml
Fecal biomarker	Calprotectin	1900 μg/g	3200 μg/g	34 μg/g
	S100A12	400 ng/ml	470 ng/ml	70 ng/ml
	Lactoferrin	1100 μg/g	440 μg/g	1 μg/g
	NGAL	6 μg/g	5 μg/g	0.3 μg/g
	MPO	100 μg/g	60 μg/g	4 μg/g
	MMP-9	6 ng/ml	2 ng/ml	0.6 ng/ml
	IAP	20% less	22% less	--
Urine biomarker	MMP-9[214]	95%	91%	20%
	MMP-2[214]	90%	89%	25%
	MMP9/NGAL[214]	90%	86%	3%

* Speed

Table 4.

miRNA biomarkers present in blood and feces used for diagnosis of IBD.

Disease type	Tissue	Associated miRNAs	Reference
CD	Peripheral blood	↑: miR-1991-5p, miR362-3p, miR-340*, miR532-3p, miRplus-E1271 ↓: miR-149*, miRplus-F1065	215
UC	Peripheral blood	↑: miR-28-5p, miR-151-5p, miR-199a-5p, miR-340*, miRplus-E1271, miR-103-2*, miR-362-3p, miR-532-3p, miR-3180-3p, miRplus-E1035, miRplus-F1159 ↓: miR-505*	215
CD	Serum	↑:miR-140-3p, miR-877, miR-127-3p ↓:miR-150	216
UC	Serum	↑: miR-140-3p, miR-29a, miR-196b, miR-127-3p ↓: miR-150	216
CD	Serum	↑: miR-146b-5p	217
UC	Serum	↑: miR-146b-5p	217
CD	Serum Feces	↑: miR-16, miR-21, and miR-223 ↑: miR-223, miR-155	218
UC	Serum Feces	↑: miR-16, miR-21, and miR-223 ↑: miR-223, miR-155	218
CD	Feces	↑: miR-21, miR-106a, miR-96, miR-203, miR-20a, miR-326, and miR-92 ↓: miR-320, miR-126, miR-484-5p, miR-143, miR-145, miR-16, and miR-125b	219
UC	Feces	↑: miR-21, miR-203, miR-126, and miR16 ↓: miR-320 and miR-192	219
CD	Feces	↑: miR-223 and miR-1246	220
UC	Feces	↑: miR-223 and miR-1246	220
CD	Feces	↑: miR-135b, miR-223 and miR-451	221
UC	Feces	↑: miR-221 and miR-18a	221

↑= increased expression, ↓= decreased expression