REVIEW OF LABORATORY BIOMARKERS OF INFLAMMATORY BOWEL DISEASE

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ABSTRACT

Inflammatory bowel disease (IBD), a cluster of chronic, immune–related and long–term disorders that cause fever, abdominal pain/cramping, fatigue, severe and recurrent diarrhoea, rectal bleeding and weight loss, and characterised by inflammation of gastrointestinal tract (GIT), is categorised as crohn's disease (CD), ulcerative colitis (UC) or IBD unclassified (IBDU). Assessing the diagnosis, severity and monitoring of IBD is largely based on the combined effects of clinical presentations, endoscopy, radiology, histology and laboratory biomarkers. Some laboratory biomarkers of IBD such as C–reactive protein (CRP), erythrocyte sedimentation rate (ESR), platelets etc are associated with systemic and gastrointestinal inflammation or disease activity, i.e., active or quiescent IBD. Others are linked to genetic predisposition, e.g., autophagy genes, nucleotide–binding oligomerization domain–containing protein 2 (NOD2), interleukin–23 receptor (IL23R) etc, correlated with the neoplastic transformation, e.g., M2–pyruvate kinase, miRNAs, mucosal chitinase–3–like protein 1 (CH13L1) etc, and coupled to drug metabolism in relation to therapeutic intervention e.g., Thiopurine Methyltransferase (TPMT) and 6–Thioguanine Nucleotide (6TGN). There are also those such as anti–neutrophil cytoplasmic antibodies (ANCAs), and anti–saccharomyces cerevisiae antibodies (ASCAs) that are related to the type of IBD, i.e., either CD, UC or IBDU. This review summarises the characterisation of laboratory biomarkers of IBD including a narrative on their future perspectives from the standpoint of diagnosis, prognosis, monitoring the disease course and a practical algorithm for the practical application of these biomarkers in the assessment of IBD.

Keywords: Biomarker, Inflammatory Bowel Disease, Crohn's Disease, Ulcerative Colitis, S100 Proteins, Calprotectin, S100A12, C–reactive Protein.

INTRODUCTION

Inflammatory bowel disease (IBD), a cluster of chronic, immune–related and long–term disorders that cause fever, abdominal pain/cramping, fatigue, severe and recurrent diarrhoea, rectal bleeding and weight loss, and characterised by inflammation of the gastrointestinal tract (GIT), is categorised as crohn's disease (CD), ulcerative colitis (UC) or IBD unclassified (IBDU).[1–3]

Epidemiologically, IBD has witnessed considerable changes with consequent annual variations in the incidence by geographical region and increase in the disease burden globally. With worldwide prevalence at ≈5 million, the incidence per 100,000 population in Europe stood at ≈10.5–46.1%, Middle East at ≈23.7–39.8%, North America at ≈7.3–0.2%, Oceania at ≈0.21–3.67% and Asia at ≈1.37–1.5% , there appears to be an increasing trend with age in the disease burden in adults (exceeding 1% in those >70 years) relative to children and adolescents, and in female (≈3.9 million) compared to male (≈3.0 million) population.[1–3]

The advent of more convenient, available and affordable laboratory biomarkers for the diagnosis, prognosis and treatment of IBD is expected to totally change the way that patients presenting with the disease are treated[1]. Although the recent scientific and technological advances in the development, optimisation and analytical validation of immunoassay kits have somewhat eased the practical issues associated with the application of laboratory biomarkers in the assessment of IBD, there still exist some uncertainties over the gains recorded with the advances in genomic, proteomic and metabolics arrays in relation to clinical laboratory testing[1].

Recent advances in immunoassay technologies have necessitated the categorisation of these laboratory biomarkers of IBD into those biomarkers that are associated with systemic and gastrointestinal inflammation including disease activity, i.e., active or quiescent IBD; those biomarkers that are linked to genetic predisposition of IBD; those biomarkers that are correlated with the neoplastic transformation of IBD; those biomarkers that are coupled to drug metabolism in relation to therapeutic intervention of IBD; and those biomarkers that are related to type of IBD, i.e., either CD, UC or IBDU.[1–3]

More information may be needed on the many aspects of laboratory biomarkers to diagnose IBD, differentiate between disease states (CD vs. UC), evaluate disease activity (active vs. quiescent), confirm disease sites or locations as in small intestine: ileal and upper (A1) vs. colonic and ileo–colonic (A2); large intestine: proctitis (E1) vs. distal colitis (E2) vs. pancolitis (E3), predict the disease course and relapse, and monitor follow–up and response to treatment.[1]

The diagnosis, severity and monitoring of IBD are usually based on a combination of clinical presentations, endoscopy, radiology, laboratory biomarkers and, if appropriate histology. Laboratory biomarkers should be non–invasive, cheap, simple, objective, rapid, easy to perform and reproducible *(table 1)*. An ideal biomarker for IBD that would combine all these characteristics is not available.[1,2] Serum biomarkers of systemic inflammation are shown in *table 2* and faecal biomarkers of gastrointestinal inflammation are shown in *table 3*.

Faecal biomarkers may be regarded as the 'gold standards' in intestinal inflammation. They are however, limited by certain factors including

lack of specificity for IBD; lack of validated, optimal and varying assay cut–offs make it difficult to characterize active inflammatory disease, distinguish IBD from irritable bowel syndrome (IBS), predict clinical remission and mucosal healing, and assess response to treatment; levels of calprotectin in stool samples are dependent on diverse physiological considerations such as age and clinical comorbidities; significant overlap in faecal calprotectin levels (50–150 µg/g) in both IBD and IBS patients presents an ambiguous situation regarding the decision to refer a patient to endoscopy or not, and intra–individual and inter–individual variability, spot variability in the same sample and reluctance of some patients to provide stool samples.[1–3] These issues may be overcome by using serum samples for the measurement of calprotectin and S100A12[1]. This offers the prospect that serum calprotectin and serum S100A12 could replace or supplement faecal calprotectin and faecal S100A12 in the identification and assessment of IBD.

Whilst it is important for laboratory biomarkers of IBD to be non–invasive, cheap, simple, objective, rapid, easy to perform and reproducible, the 'real–world' experience with these biomarkers is increasing together with the increasing number of patients with IBD for which the usefulness of these laboratory biomarkers to assess the disease have become necessary.[1–3] The 'ideal biomarker' that would integrate all the preceding characteristics for evaluating the analytical performance and diagnostic accuracy of these laboratory biomarkers in IBD is not available.[1] Therefore, the search for the availability of a single laboratory biomarker that would demonstrate the attributes of an 'ideal biomarker' for use in assessing IBD continues.

This objectives of this review were to present a summary of the characterisation of laboratory biomarkers of IBD including a narrative on their future perspectives from the standpoint of diagnosis, prognosis and monitoring the disease course. A practical algorithm for the practical application of these biomarkers, both current and potential future biomarkers, in the assessment of IBD will also merit discussion. A conclusion will be added.

A. Laboratory biomarkers with widespread application

1. Serum C–reactive protein and cytokines

C–Reactive Protein (CRP), a pentameric protein synthesized predominantly in the liver but also in vascular walls and adipose tissue, is a well–established biomarker of acute inflammation. During acute phase response, hepatocytes rapidly increase production of CRP under the influence of interleukin–6 (IL–6), tumour necrosis factor–alpha (TNF–α), and interleukin–1β (IL–1β). Serum CRP, therefore, rises rapidly up to 100 to 1000–fold, peaking in two days and then decreases rapidly with the resolution of the acute insult as it has a half–life of 19 hours. Functionally, CRP is recruited into the complement activation process when it binds to organisms or particles that contain phosphocholine which enables it to opsonise infectious agents and damaged cells.[4–6] In healthy individuals, CRP circulates in low concentration (about 1 mg/L). CRP concentrations around 10 to 40 mg/L may be seen in chronic inflammation.[7,8]

CRP is generally increased in IBD, but appears to respond differently to inflammation in UC and CD. A rise in serum CRP level correlates well with the disease activity in CD. CRP, however, may not be raised or mildly raised in

the presence of increased disease activity in UC even though increased levels of IL–6, IL–1β or TNF–α are observed.[9] Other inflammatory markers such as β_2–microglobulin correlate better with histology scores in UC.[10,11] The main reason given for this differential CRP response in IBD is that inflammation is limited to the mucosa in UC and, less likely to provoke a systemic response to inflammation when compared to transmural inflammation in CD. Other possible reasons include increased IL–6 levels in CD compared to UC[12] and CRP gene polymorphism in UC and CD.[13–15] CRP levels in IBD patients, however, are not associated with CRP gene polymorphism.[16]

Table 1 – Characteristics of an ideal biomarker for use in the assessment of IBD[1]

Performance	Criteria Characteristics
Simple	Disease specific: Identify individuals with IBD; Able to differentiate IBD from non–IBD cases; Able to predict the remission or relapse; Monitor the effect of treatment; Prognostic value in assessing morbidity/mortality
Ease of performance	Ability to objectively measure disease activity without ambiguity
Invasiveness	Not invasive or minimally invasive
Cost	Affordable
Rapid	Quick turnaround time
Reproducibility	Assay results not showing discrepancies between individuals and clinical laboratories.

Table 2 – Serum acute phase proteins and their responses to IBD and Other inflammatory processes[1]. *α_2–macroglobulin shows a different response in animals in comparison to humans, both in positive and negative acute phase protein respectively.

Acute Phase Protein	Increased (↑)	Decreased (↓)
Proteinase Inhibitors	α_1–Antitrypsin, α_1–Antichymotrypsin, α_2–Macroglobulin*	
Coagulation and Fibrinolytic Proteins	Fibrinogen, Prothrombin, Factor VIII, Plasminogen, Tissue Plasminogen Activator Antithrombin	Factor XII
Complement System	C1s, C2, C3, C4, C5, C1 Inhibitor, C9	Albumin, Transferrin
Transport Proteins	Haptoglobin, Haemopexin, Caeruloplasmin	Insulin–like Growth Factor, α– Fetoprotein, Cholinesterase
Other Acute Phase Proteins	C–reactive protein, Serum Amyloid A, Ferritin, Fibronectin, Orosomucoid (α_1–Acid Glycoprotein).	

Table 3 – Faecal biomarkers in clinical use for the diagnosis and differential diagnosis of IBD including its discrimination from IBS[3].

Faecal Biomarker	Major Source or Origin
Calprotectin (S100A8/S100A9)	Neutrophils, Monocytes and Epithelial Cells.
Calgranulin C or EN–RAGE (S100A12)	Neutrophils
Lactoferrin	Mucosal Epithelial Cells and Neutrophils
M2–Pyruvate Kinase (M2PK)	Expressed by rapidly dividing cells
Neopterin	Activated Macrophages
Metalloproteinases (MMPs)	Different Cell types including Activated Neutrophils
Myeloperoxidases (MPOs)	Activated Neutrophils
Polymorphonuclear Elastase (PMN)	Activated Neutrophils

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The utility of CRP as a serum biomarker in the assessment of IBD is still unclear. CRP is useful in assessing IBD patients with the active disease. However, there is the disadvantage that the inclusion of CRP in IBD assessment carries the increased risk of being restricted to IBD patients with high concentration of the protein.[1] Although the application of CRP may offer the promise of an increased prospect of response to treatment with a reduced rate for placebo considerations, it is however, important to emphasise the fact that the United States Food and Drug Administration (FDA) placed restrictions on using CRP in IBD assessment.[1] CRP presents a fair evaluation of the follow–up effect of therapeutic intervention in IBD patients. The ambiguity of CRP cut–off values in assessing IBD makes the utility of CRP very problematic.[1]

2. Circulating haemopoietic biomarkers: erythrocyte sedimentation rate (ESR), platelet count, mean platelet volume (MCV) and red cell distribution width (RDW)

ESR is an indirect measure of systemic inflammation. ESR measures the rate of migration of erythrocytes through the plasma, and is increased in the presence of increased proteins including acute phase proteins. ESR, compared to CRP, is slow to peak in response to inflammation and slow to decline after resolution of inflammation and is therefore of limited value in IBD assessment.[1,17]

Inflammation processes, including IBD, cause an increase in platelets and changes in their morphology. Platelet count increases in patients with IBD, particularly in UC when reticulated platelets are taken into account.[18,19] It, however, is not a useful biomarker of IBD, given the wide range of a normal platelet count.[1,20]

MPV has been reported as decreased in active IBD and has a negative correlation with CRP, while other studies have found no correlation between a fall in MPV and the disease activity.[21,22] Likewise, leucocytes lack specificity as a biomarker for IBD,[23] and are influenced by treatments in IBD with drugs such as glucocorticoids or azathioprine (increased) and 6–mercaptopurine (decreased).[1]

RDW is a measure of size variability and heterogeneity of erythrocytes in peripheral blood. RDW increases in active IBD, and particularly in CD compared to UC. RDW at a diagnostic cut–off value of 13.8% in non–anaemic UC patients has a sensitivity and specificity of 76% and 86% respectively, and in non–anaemic CD patients at a diagnostic cut–off value of 14.1% has a sensitivity and specificity of 82% and 83% respectively.[23,24]

3. Other serum acute phase proteins

Other acute phase reactants (Table 1.3) such as fibrinogen, sialic acid, α_1–acid glycoprotein (orosomucoid), α_1–antitrypsin, α_2–globulin, β_2–microglobulin, serum amyloid A (SAA) and albumin as biomarkers of IBD have not been widely studied because of their apparent inferiority to CRP. α_1–acid glycoprotein, shows good correlation with IBD disease activity but its half–life of 5 days makes it unsuitable as IBD biomarker.[1,17]

4. Faecal lactoferrin

Lactoferrin is an iron–binding protein found in neutrophil granulocytes. In acute intestinal inflammation, increased mucosal infiltration of neutrophils and subsequent secretion of lactoferrin into the intestinal tract results in increased faecal lactoferrin.[25,26] Lactoferrin has anti–bacterial activity by limiting availability of iron, and causes direct damage to bacterial cell membrane. Faecal lactoferrin is resistant to degradation and proteolysis, although less so than calprotectin, making it a useful biomarker of intestinal inflammation. The significant proportion of faecal lactoferrin in stool is, therefore, a reflection of intestinal inflammation.[27–29]

Lactoferrin is stable in stool for up to 5 days, does not deteriorate with repeated freezing and thawing, and can be quantitatively measured by the enzyme–linked immunosorbent assay (ELISA) technique. It is a non–specific intestinal biomarker being raised not only in active IBD but in other inflammatory intestinal disorders including infective diarrhoea, colon cancer and non–steroidal anti–inflammatory drugs (NSAIDs) enteropathy. Faecal lactoferrin correlates well with the clinical, endoscopic and histological grading of IBD disease activity and therefore, may distinguish between active and inactive IBD, and between IBD patients and healthy controls.[26,30–36] For predicting relapse, faecal lactoferrin has 46% sensitivity and 61% specificity for UC, and 77% sensitivity and 68% specificity for CD.[37] Comparable diagnostic accuracy of lactoferrin and calprotectin exist in patients with IBD and in patients with irritable bowel syndrome (IBS) and healthy controls[38]. Calprotectin and lactoferrin had similar sensitivity (78% vs. 80%), specificity (83% vs. 85%), overall diagnostic accuracy of 80% vs. 81% and are both significantly elevated in children with IBD.[38,39]

5. Faecal neopterin

Neopterin is a metabolite of biopterin released by activated macrophages and monocytes. Faecal neopterin correlates well with disease activity in IBD particularly with the severity of mucosal lesions. Faecal neopterin shows better correlation with endoscopic scores in UC (r = 0.72; $p < 0.0001$) than in CD (r = 0.47; $p < 0.0001$). Faecal neopterin is significantly higher in clinically and endoscopically active IBD compared to inactive IBD. The diagnostic accuracy of faecal neopterin to predict endoscopic activity in IBD compares favourably with faecal calprotectin in CD (74%) and in UC (87%) and, like calprotectin and lactoferrin, release of neopterin is non–specific as it could be triggered in response to disease conditions of viral infection and cell–mediated immune response during the early phase of inflammation.[40,41]

6. Faecal metalloproteinases (MMPs)

MMPs are zinc–dependent endopeptidases secreted by various cell types. MMP–9 is the MMP released in highest concentrations by activated neutrophils during intestinal inflammation such as in IBD. While MMP–1, MMP–2 and MMP–3 are detected in significantly higher amount in UC, only MMP–9 levels are significantly higher in active UC than in IBS or healthy controls thereby making it a reliable biomarker in distinguishing between UC and IBS based on 85% sensitivity and 100% specificity.[42] Faecal MMP–9 correlates well with other faecal biomarkers including calprotectin, lactoferrin and neopterin in endoscopically assessed disease activity in IBD and also demonstrates comparable diagnostic accuracy with faecal calprotectin.[43]

7. Faecal myeloperoxidases (MPOs)

MPOs are lysosomal proteins released by activated neutrophils in response to inflammation. The diagnostic accuracy of faecal MPOs to assess endoscopic disease activity in IBD is inferior to calprotectin and polymorphonuclear (PMN) elastase.[44]

8. Faecal polymorphonuclear (PMN) elastase

PMN elastase is released by activated neutrophils during inflammation, and is stable for up to 4 days in stool at ambient temperature. Faecal PMN elastase may be a useful biomarker in IBD as it is significantly higher in patients with active IBD compared to inactive IBD. This is reflected in its diagnostic accuracy of 84% sensitivity and 87% specificity for IBD that increases to 96% sensitivity and 100% specificity when combined with calprotectin and lactoferrin.[35,45]

9. Faecal M2–pyruvate kinase (M2PK)

Faecal levels of M2PK, a multi–functional protein found in undifferentiated and proliferating cells, are associated with active IBD, and correlate well with calprotectin in distinguishing IBD from IBS. M2PK is a superior biomarker to calprotectin, lactoferrin and S100A12 in predicting steroid refractoriness in severe paediatric UC. It also holds promise as a potential screening biomarker for colorectal cancer (CRC) in UC.[46–49]

10. The S100 Proteins: Faecal and serum S100A12

The family of low molecular weight S100 proteins or calgranulins *(figure 1)* have several functions including roles in cellular inflammation, proliferation, differentiation, apoptosis, signal transduction, calcium homeostasis and energy metabolism.[50–53] In chronic inflammation, the S100 protein family

are actively expressed in activated granulocytes.[54–56]

The cytokine–like extracellular functions of S100 proteins such as chemotactic activities related to inflammation and the acute phase response are exhibited mainly by S100A8 (calgranulin A), S100A9 (calgranulin B) and S100A12 (calgranulin C), and these are commonly referred to as the calgranulins or myeloid–related proteins (MRP).[57–59] The name 'S100 proteins' was derived from their ability to be 100% soluble in a saturated solution of ammonium sulphate at neutral pH. These proteins bind calcium, and are characterized by two calcium–binding motifs called the elongation factor (EF) hand. There are over 600 members of EF–hand super family.[60–63] The S100 proteins are expressed in myeloid cells including neutrophils, monocytes, and dendritic cells.[64]

S100A12 *(figure 2)* is a 10.4 *kilodalton (kDa)* molecular weight, 91–amino acids protein. It is a calcium–binding, proinflammatory protein predominantly expressed and secreted by neutrophil granulocytes, and represents 5% of total cytosolic protein.[65,66] S100A12 is a ligand for the receptor for advanced glycation end products (RAGE) and therefore, also named as extracellular newly identified receptor for advanced glycation end products binding protein (EN–RAGE). Other alternative names for S100A12 are calgranulin C, migration inhibition factor–related protein 6 (MRP–6) and calcium–binding protein in amniotic fluid (CAAF–1).[57,67,68] The binding of S100A12 to RAGE modulates these extracellular functions in disease. In inflammatory disease, serum S100 correlates with disease activity parameters and in these conditions S100 proteins may be superior to conventional laboratory biomarkers of inflammation including CRP and ESR.[69–71]

Serum S100A12 have been reported in increased concentrations in various biological materials including synovial fluid, synovial tissue and serum of patients with inflammatory arthritis.[72–75] Serum S100A12 is also raised in neurodegenerative diseases, diabetes mellitus, rheumatoid arthritis, osteoarthritis, cancerogenesis, familial mediterranean fever, idiopathic pulmonary fibrosis, cardiovascular diseases and atherosclerosis.[76–82]. S100A12 levels were also elevated in patients with peripheral radiographic features ($p = 0.036$), but did not correlate with clinical variables of disease activity in psoriatic arthritis.[83] In haemodialysis patients, levels of S100A12 are linked to cardiovascular disease.[84,85] Elevated concentrations of S100A12 and its receptors are found in pulmonary tissue and broncho–alveolar lavage fluid in acute lung injury.[86,87] Both S100A8 and S100A9 hetero–complexes are actively expressed preceding prostate tumour genesis and subsequent development, progression and enlargement of prostate carcinomas. Alone, serum S100A9 is increased significantly in prostate cancer patients compared to healthy controls or patients with benign prostatic hyperplasia (BPH); thereby underlining its role as a useful biomarker in differentiating prostate carcinoma and BPH.[88]

Faecal S100A12 have been shown to be a novel non–invasive biomarker of IBD in paediatric populations.[89,90] S100A12 levels in stool can be used as an indicator of disease activity in chronic IBD and to gauge the degree of gastrointestinal tract inflammation. As a biomarker of neutrophil activation, faecal S100A12 could play a significant role as a non–invasive biomarker of intestinal inflammation.

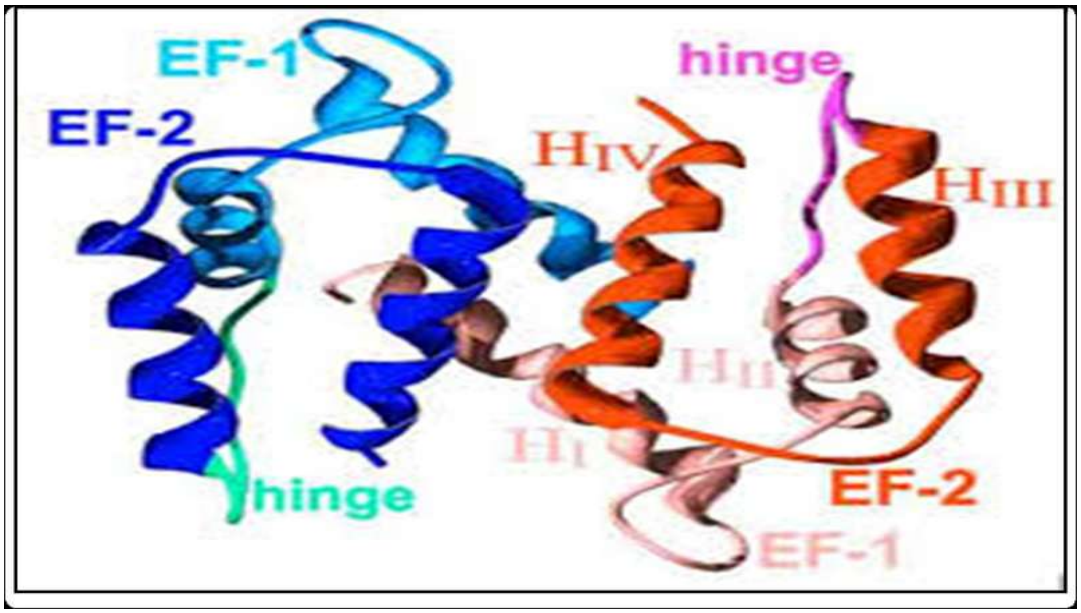

Figure 1 – Dimer structure of S100 protein. S100 proteins are small proteins with a molecular weight of 10 – 12 kDa. Each S100 protein consists of two EF–hand helix–loop–helix structural motifs, which are arranged in a back–to–back manner and linked with a flexible hinge.[95]

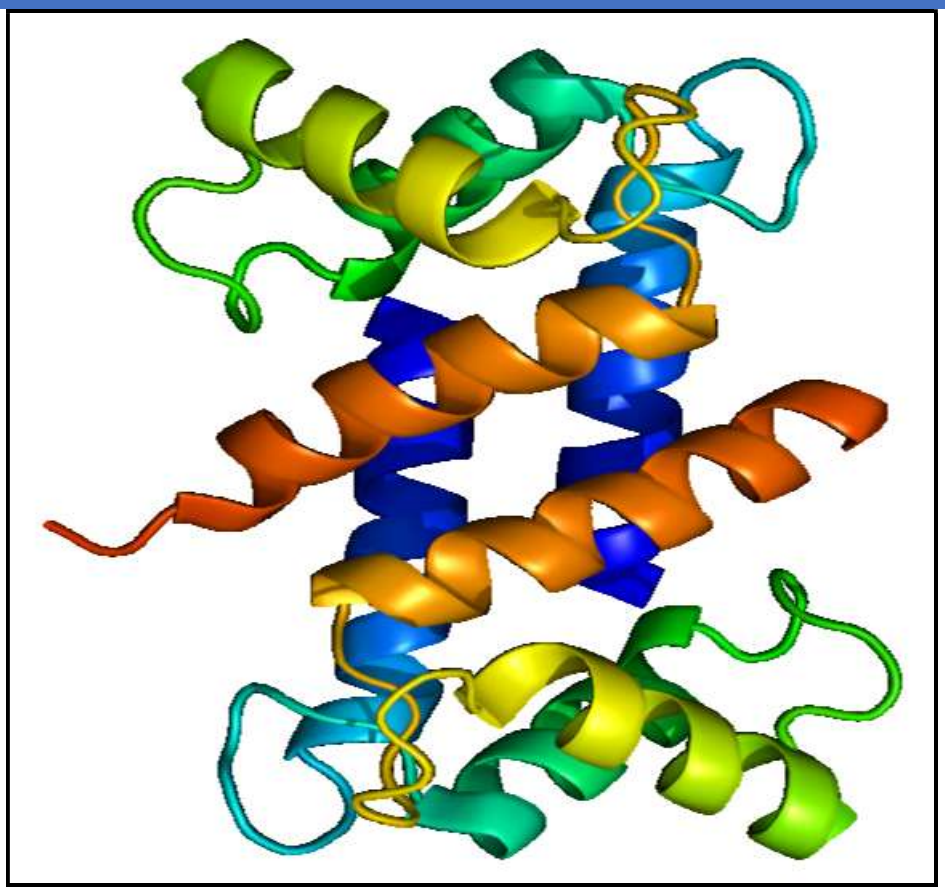

Figure 2 – Structure of S100A12. A three–dimensional model of the crystal structure of the S100A12 protein in a hexameric form and its proposed role in receptor signalling. The stereo view of the Ca^{2+} and Cu^{2+}–S100A12 dimer in ribbon representation, with individual subunits shown in red and blue. The chain topology, subunit arrangement, and juxtaposition of metal–binding sites are typical of metal–bound S100 proteins.[91–94]

11. Faecal and serum calprotectin

Calprotectin *(figure 3),* is a 36 kilodalton (kDa) calcium– and zinc–binding protein composed of two heavy 14 kDa S100A9 (MRP14) and one light 8 kDa S100A8 (MRP8) subunits which are members of the EF–hand motif containing S100 family of proteins. Like all S100 proteins, the genes that code for calprotectin are located within the gene cluster on chromosome 1q21 region. Calprotectin is expressed predominantly in neutrophils and monocytes – in which it constitutes up to 60% of the cytosolic protein. It is also expressed in macrophages, keratinocytes, epithelial cells and endothelial cells.[50,96-100]

Calprotectin was first characterised in 1980 as the Leucocyte protein candidate 1 (L1), to reflect *in–vivo* granulocyte turnover.[101-104] L1 was renamed calprotectin in recognition of its antimicrobial activity[105], calcium–binding roles and subsequent involvement in intracellular signal transduction and regulatory functions in acute phase response and inflammatory processes[96,106,107]. Neither S100A8 nor S100A9 subunits in isolation have anti–microbial

characteristics. It is possible that the high affinity of calprotectin subunits, S100A8 and S100A9, for zinc–binding site could explain the reduction of zinc concentration sufficiently to allow calprotectin to inhibit microbial growth.[106,108-111]

Alternative names for calprotectin include: MRP–8 and MRP–14 (MRP8/MRP14), p8/14, p34 and S100A8/S100A9. Calprotectin is composed of two heterocomplexes: S100A8 (also known as MRP–8, Calgranulin A and CP–10 in mouse) and S100A9 (also known as MRP–14 and Calgranulin B).

Calprotectin is found in plasma, saliva, cerebrospinal fluid, urine and faeces. Circulating calprotectin is commonly raised in inflammatory diseases including acute coronary syndromes, cystic fibrosis, multiple sclerosis, human immunodeficiency virus, rheumatoid arthritis, reactive arthritis, juvenile chronic arthritis, juvenile idiopathic arthritis, psoriatic arthritis, polymyalgia rheumatica, systemic lupus erythematosus and acute rejection in kidney allograft transplantation[103,112–130]

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Calprotectin expression correlates with microglial activation in cerebral malaria; and serum levels are prognostic biomarkers in recurrent infection and survival in alcoholic liver disease.[131-133] Increased calprotectin concentrations in faecal samples of patients with IBD that reflect granulocyte migration through the inflamed intestinal wall has been reported.[134-136] Measurement of faecal calprotectin now enjoys wide application in the diagnosis and monitoring of IBD, and an increased concentration indicates organic intestinal disorders. Calprotectin is an extremely stable protein, and remains unaltered in stool samples left unprepared for longer than seven days[137-139]. Studies have focused on the accuracy of faecal calprotectin in the diagnosis and monitoring of IBD. A meta–analysis reported 95% sensitivity and 91% specificity of faecal calprotectin in the identification of IBD. This study also reported that faecal calprotectin was superior to CRP and ESR[140].

The United Kingdom's National Institute for Health and Care Excellence (NICE) recommends faecal calprotectin as a diagnostic tool to help in the differential diagnosis of IBD and IBS[141]. A normal faecal calprotectin excludes IBD, whereas an elevated faecal calprotectin is an indication for colonoscopy, thereby reducing referrals for unnecessary endoscopic evaluation. A meta–analysis of 13 studies concluded that faecal calprotectin testing would result in a 67% reduction in the number of adults requiring endoscopy, but with a delayed diagnosis in 8% of adults because of false negative results[142].

One area of controversy surrounding faecal calprotectin testing is the determination of an appropriate cut–off value, above which the result is deemed as positive. In most centres, a relatively low level of 50 µg/g is used. A cohort of adult patients undergoing faecal calprotectin testing in primary care was studied[143]. At a cut–off value of 50 µg/g, faecal calprotectin had a negative predictive value (NPV) of 98% and positive predictive value (PPV) of 28%. Increasing the cut–off value to 150 µg/g gave a very comparable NPV of 97%, but a much

higher PPV of 71%. Given these values, it was calculated that increasing the cut–off value to 150 µg/g, would reduce colonoscopy and flexible sigmoidoscopy bookings by 10% at the cost of 4 missed cases of IBD[143].

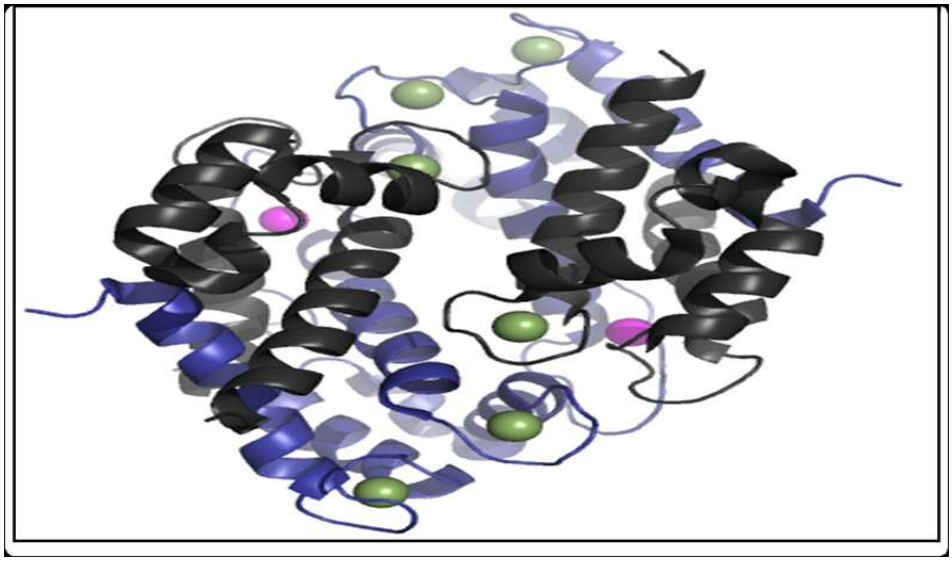

Figure 3 – Structure of calprotectin. A three–dimensional model of the crystal structure of Mn^{2+} and Ca^{2+} loaded calprotectin protein, showing two heterodimers: S100A8 and S100A9 as determined by X–ray diffraction. The grey and blue chains represent S100A8 and S100A9, respectively. The purple spheres represent Mn^{2+} and green spheres represent Ca^{2+}. Only one manganese ion can bind per calprotectin heterodimer. The modified figure was taken from the protein data bank (PDB) at: http://www.rcsb.org/pdb

12. Blood autophagy genes and nucleotide–binding oligomerization domain–containing protein 2 (NOD2)

Family history of CD is a risk factor for CD[144]. Genome–wide association studies have reported that NOD2 and Autophagy genes are associated with CD risk while IL–23/IL–17 is associated with increased risk of both CD and UC[145].

13. Serum anti–neutrophil cytoplasmic antibodies (ANCAs) and anti–saccharomyces cerevisiae antibodies (ASCAs)

It may be difficult to differentiate CD from UC due to their overlapping pathological, endoscopic and clinical features. Biomarkers like ANCAs, which are antibodies against granules of neutrophil cytoplasm and ASCAs, which are antibodies against mannan found in the cell walls of saccharomyces cerevisiae (S. cerevisiae) have been used to help differentiate CD from UC[146].

The sensitivity and specificity of perinuclear ANCA (pANCA) for the diagnosis of ulcerative colitis is 63% and 86% respectively. ASCAs for

diagnosing CD had a sensitivity of 72% and a specificity of 82%[147]. The utility of these serological tests in differentiating UC from CD is therefore limited but may be of value in studying disease heterogeneity and disease epidemiology.

Other serologic tests investigated to improve the diagnosis and differential diagnosis of IBD include: antibodies to Escherichia coli outer membrane porin C (anti–OmpC), antibodies against laminaribioside (ALCAs), antibodies against chitobioside (ACCAs), Pseudomonas fluorescens–associated sequence 12, antibodies to mannobioside (AMCAs), pancreatic autoantibodies (PAB), and anti–flagellin CBir1 but all are of limited diagnostic utility.[148-153]

B. Laboratory biomarkers with limited application

The foregoing biomarkers enjoy widespread application in IBD assessment. However, there are some that are not extensively used, or those whose utility for efficacy in IBD assessment can be enhanced only when in combination with one or more biomarkers of similar or different biochemical composition.[49]

These include: adenosine deaminase (ADA), mopterin, nitric oxide, substance P, micro ribonucleic acids (MicroRNAs), lipopolysaccharide–binding protein (LPBP) and cluster of differentiation (CD14), abnormal lectin–based immunoglobulin G (IgG) glycosylation, soluble triggering receptor expressed on myeloid cells–1 (sTREM–1), Soluble suppression of tumorigenicity 2 (ST2), Quantitative fecal immunochemical test (QFIT), activated thrombin activatable fibrinolysis inhibitor (TAFIa), chitinase 3–like–1, mucosal cytokine, mucosal indoleamine 2,3 dioxygenase–1 (IDO1) and angiogenin[49].

Others are: genes, antibodies toward infliximab, mucosal CHI3L1, urine salicylate level, and thiopurine methyltransferase and 6–thioguanine nucleotide (TPMT)[49].

14. Inflammatory and serological biomarkers in differential diagnosis of inflammatory bowel disease

The differential diagnosis of IBD includes colon cancer, ischaemic colitis, diverticulitis, bacterial and viral infection. These alternatives or co–existing disorders must be ruled out prior to confirmation of intestinal inflammation as a first step in the differential diagnosis of IBD *(Figure 4)*.

The likelihood of CD, UC and Moderate/High Clinical Suspicion are subjected to further radiographic, endoscopic and histological investigations to complete the differential diagnosis. While the above illustration *(figure 4)* provides the necessary information to aid in test selection, interpretation, diagnosis and overall patient management decisions, it is not a substitute for the clinician's underpinning knowledge of IBD in patient assessment based on clinical expertise.[154-157]

Accurate diagnosis of CD and UC is therefore dependent on not only laboratory test results but also on the patient's clinical history and examination, histology, imaging results (X–ray, CT and/or MRI scans) and endoscopy[154,158].

Inflammation in UC is relatively superficial because it affects the mucosa. It is confined to the colon starting at the rectum, with rare extension to the terminal ileum. There is, however, a 10 to 15% chance of misdiagnosis due to difficulty in distinguishing UC from CD. Accuracy in differential diagnosis is critical because while inflammation in CD extends deeper into the tissues and could affect any portion of the gastrointestinal tract, associated

symptoms like abdominal pain, fever, malnutrition and severe bloody diarrhoea are common to both[159].

15. Inflammatory biomarkers in differential diagnosis of inflammatory bowel disease

The initial step in the identification of IBD is exclusion of other organic disorders. Serum CRP, ESR, faecal calprotectin and faecal lactoferrin form the available inflammatory biomarkers.

Many laboratory biomarkers have been evaluated as the ideal replacement for, or supplement to faecal calprotectin in IBD studies but CRP and ESR remain the most widely used and assessed tests[1]. Other biomarkers used include leucocyte counts, platelet counts, albumin levels and orosomucoid concentrations[160], but there is no evidence that they are of greater benefit compared to CRP in the identification of IBD and monitoring its disease activity[161].

CRP is routinely available as it is rapid, inexpensive and a simple testing technique. CRP's reliability as a biomarker of choice in routine clinical practice is well established[162]. Serum CRP may help in differentiating IBD from other functional bowel disorders[163]. However, normal CRP levels (≤ 5 mg/L) at diagnosis occur in majority of UC patients and in 25% of CD patients.[164] There is, however, a correlation between CRP and endoscopic activity in IBD and this may have a useful role in monitoring responses to therapy.[165,166]

In summary, increased CRP is more common in CD than in UC, thereby supporting CRP to be a useful biomarker of disease activity in CD than in UC.[1,164,165] CRP, however, like most serologic biomarkers, is a non–specific biomarker of systemic inflammation.[1]

16. Serological biomarkers in differential diagnosis of inflammatory bowel disease

In the presence of inflammation, ANCAs and ASCAs may be useful in the identification of IBD.[154,155] A positive result will necessitate radiology, endoscopy and histological investigation to confirm the diagnosis of IBD and differentiation of CD and UC. If serological markers are negative in the presence of a high clinical suspicion of IBD, then radiologic, endoscopic and histological findings could be used to diagnose or exclude IBD and, if present, to distinguish CD and UC.

ASCAs and atypical pANCA are the two serologic markers most commonly used to distinguish CD and UC.[167] A positive ANCAs result is reflexed to determine the relevant pattern(s): cytoplasmic (cANCA), perinuclear (pANCA) or atypical pANCA patterns and their titres. Whereas cANCA and pANCA are found in vasculitis, atypical pANCA is present in IBD. While atypical pANCA is detected in only 5% to 25% of patients with CD, it occurs in about 55% to 80% of those with UC.[157,168] Conversely, ASCAs is detected in about 60% to 70% of CD patients and only in about 6% to 15% of UC patients.[168,169]

A combination of pANCA and ASCAs has a sensitivity of 53% and specificity of 93% for both CD and UC; and may be of value in assessing IBD in patients that cannot be distinguished as CD or UC on the basis of indeterminate colitis or established criteria.[156,157]

Atypical pANCA and ASCAs may help stratify CD. Positive atypical pANCA in CD indicate colonic involvement and an association with a clinical phenotype analogous to UC or UC–like CD, while a positive ASCAs result is linked with the non–UC–like CD.[169,170]Serologic biomarkers may be of value in children. In children at high risk of IBD, pANCA and

ASCAs may help to identify those children with IBD and to avoid invasive assessment.[157,171-174]

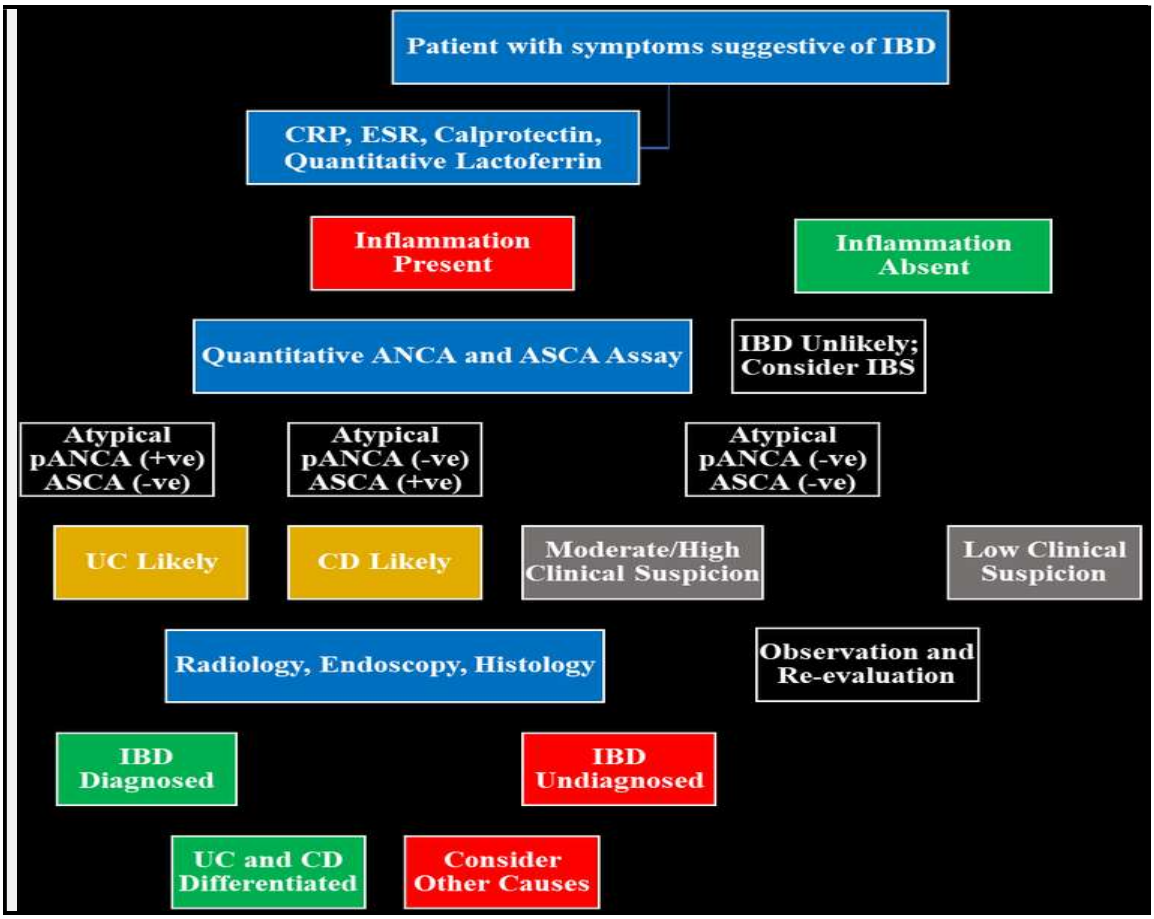

Figure 4 – The interplay of inflammatory (CRP, ESR, Calprotectin and Lactoferrin) and serological (pANCA and ASCA) biomarkers in the differential diagnosis of inflammatory bowel disease.

Management of disease activity in inflammatory bowel disease

Achieving mucosal healing with subsequent improvement in natural course of the disease state in patients with IBD remains the overall goal of treatment, including the use of immunomodulators and biological agents. Monitoring for efficacy of treatment and relapse of disease activity, therefore, becomes an important consideration.[175,176]

No single test procedure or examination has been able to satisfy all the necessary requirements for the clinical management of IBD patients. Assessing disease activity for the foreseeable future involves the use of laboratory tests, radiology, endoscopy, clinical examination and symptoms.[177] This should not, however,

detract from the search for a reliable, non–invasive, highly sensitive and reproducible biomarker of disease activity.[1,178]

The faecal excretion of ^{111}Indium–labelled (^{111}In) granulocytes is considered to be the 'gold standard' method for measuring the degree of neutrophilic infiltration into the intestinal mucosa in IBD and hence the disease activity particularly in patients with small bowel CD.[179,180] The unwieldy nature of radio–labelling techniques in addition to being expensive and involving exposure to radiation restricts their routine clinical use.

Endoscopic assessment of disease activity in IBD, particularly via ileo–colonoscopy is regarded as the 'gold standard' because it has the added advantage of enabling biopsy sampling for histopathological examination apart from offering the opportunity for direct mucosal visualization, endoscopic management of complications, assessing the success of various treatment regimens and predicting the course of disease.[181-187] Ileo–colonoscopy, however, is invasive, time–consuming and is limited by expense, risks of complications, patient discomfort and variation in interpretation between endoscopists.[188,189] This underscores the desirability for an easier method to monitor disease activity.

I. Active versus inactive disease state in inflammatory bowel disease

Increased serum CRP is an indication of active disease in CD patients and it compares favourably with endoscopic disease activity.[30] Endoscopic assessment should be considered in patients with UC if elevated serum CRP concentrations fail to normalise with or without underlying symptomology.[190]

In the patients with endoscopically confirmed active IBD, faecal calprotectin and lactoferrin levels correlate with endoscopic disease activity in both CD and UC patients.[30] When compared to serum CRP (49%), both faecal calprotectin (88%) and lactoferrin (82%) are more sensitive because of their ability to correlate better with colonic (proctitis: E1 vs. distal colitis: E2 vs. pancolitis: E3) than ileal (ileal and upper: A1 vs. colonic and ileo–colonic: A2) disease activity.[154,155]

The relatively high sensitivity of both faecal biomarkers (i.e., calprotectin and lactoferrin) in patients with active disease underscores their usefulness in managing patients with IBD[191] and thus avoiding endoscopy in patients with a high clinical suspicion of active disease with raised faecal biomarkers. As a corollary, a negative faecal biomarker result may not rule out active disease and therefore endoscopy may be required if clinically indicated.[191] Similarly, with CRP, endoscopic assessment should be considered in patients with UC if elevated faecal biomarkers fail to normalise irrespective of underlying symptoms.[190]

II. Relapse, remission, and disease course in inflammatory bowel disease

An increase in serum CRP levels predict relapse in disease activity in patients with CD following medically induced remission.[155,190]

Faecal biomarkers, particularly calprotectin, play a crucial role in predicting relapse in IBD particularly in UC than in CD.[192] Elevated faecal calprotectin level is a common feature in patients with CD who relapse compared to those who remain in remission over a 12–month follow–up.[193] Low faecal calprotectin levels help identify IBD patients who remain in stable remission during follow–up.[194]

Based on a cut–off value of 167 µg/g, faecal calprotectin with a sensitivity of 69% and specificity of 75% appears to be the best

biomarker for predicting IBD relapse following remission.[154,193] Faecal calprotectin levels may also be particularly valuable in predicting relapse in patients with CD who have undergone surgical resection.[154,195]

Faecal lactoferrin also appears to have a role in predicting IBD relapse. Elevated faecal lactoferrin levels have a sensitivity of 62% and specificity of 65% in predicting early disease relapse in paediatric patients.[37,193] In some paediatric patients with CD, pANCA and ASCAs levels also predict complicated disease courses.[154,155]

III. Response to therapy in inflammatory bowel disease

A normal CRP level in CD patients undergoing treatment is linked to a favourable response to therapy.[190] Whereas normalization of CRP levels may serve as a reliable biomarker to measure the response to therapy, there are currently a paucity of studies investigating the roles of faecal calprotectin, lactoferrin, pANCA and ASCAs in predicting response to therapy.

In summary, more information may be needed on the many aspects *(i.e., analytical and technical factors: sensitivity and specificity, accuracy and precision, reproducibility, calibration, limits of the blank, detection and quantitation; pre–analytical and biological factors: sample handling, patient characteristics, timing, analyte/assay stability; clinical factors and research design variables: purpose, validation, surrogate endpoint, reference ranges)* of laboratory biomarkers to diagnose IBD, differentiate between disease states (CD vs. UC), evaluate disease activity (active vs. quiescent), confirm disease sites or locations as in the small intestine: ileal and upper (A1) vs. colonic and ileo–colonic (A2); large intestine: proctitis (E1) vs. distal colitis (E2) vs. pancolitis (E3), predict the disease course and relapse, and monitor follow–up and response to treatment.

Summary and future perspectives

It is noteworthy that not all the biomarkers of IBD discovered through clinical research that could be available for routine application in clinical practice. Many of them fail the rigorous protocols of the clinical testing criteria and assay processing through assay development, optimisation and analytical validation, and therefore, are deemed not to be feasible and acceptable for use as laboratory biomarker for assessing IBD. However, for those biomarkers that meet the acceptable target criteria and 'fitness for purpose' via the analytical methods, there is also the added laborious intervening timeline of more than 10 years approximation that span from discovery to routine clinical use to consider.[196,197]

It is a common occurrence for newly discovered biomarkers to fail short of expectation in terms of good analytical performance and diagnostic accuracy during assay validation studies. Such are the demonstrable challenges that are intrinsic in the development and clinical application of biomarkers as articulated in a recent multicentre, open–label randomised controlled trial (RCT) for PRedicting Outcomes For Crohn's disease using a moLecular biomarker (PROFILE).[197]

The practical application of laboratory biomarkers in the assessment of inflammatory bowel disease was the subject of a recent study that reviewed the utility of current and potential future biomarkers *(figure 5)*.[198] The study reaffirmed the contemporary knowledge of the utility of currently available biomarkers such as CRP and faecal calprotectin to differentiate IBD from functional gastrointestinal pathology and to monitor the disease course. However, it presented such novel biomarkers as Oncostatin M, anti–integrin antibody, faecal MPOs, faecal

microRNAs and glycome profiling, as future biomarkers that could potentially address the risk stratification of individuals not presenting with the disease (i.e., pre–IBD) including screening those with asymptomatic IBD.[198]

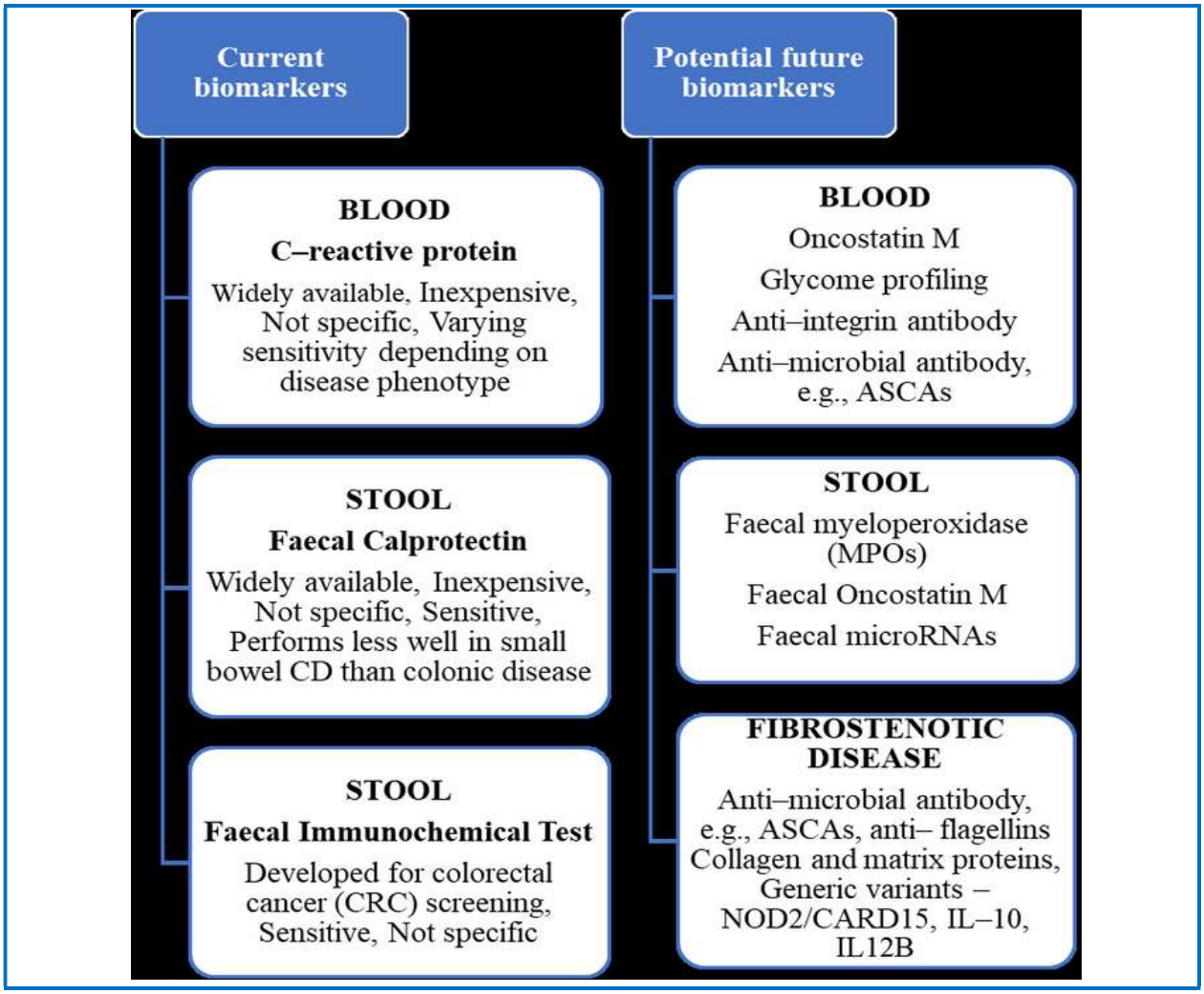

Figure 5 – An algorithm for the practical application of laboratory biomarkers in the assessment of inflammatory bowel disease. A narrative of current and potential future biomarkers for diagnosis, prognosis and disease monitoring.[198]

The future perspective on the utility of laboratory biomarkers in assessing IBD seems to tilt towards deploying the probable influence of artificial intelligence (AI) to integrate metabolomic, proteomic, genetic and transcriptomic outputs of biomarker analysis[199-201]. With the continuous growth in complexity of available data, and rapid advances in the availability and affordability of technology, the resort to AI 'to expand the horizon for biomarker

discovery, enabling the integration of multi–modal data from existing datasets to discover new biomarkers'[201] will be realised.

CONCLUSION

While it is important for laboratory biomarkers of IBD to be non–invasive, cheap, simple, objective, rapid, easy to perform and reproducible, the 'real–world' experience with these biomarkers is increasing together with the increasing number of patients with IBD for which the usefulness of these laboratory biomarkers to assess the disease have become necessary. The 'ideal biomarker' that would integrate all the preceding characteristics for evaluating the analytical performance and diagnostic accuracy of these laboratory biomarkers in IBD is not available. Therefore, the search for the availability of a single laboratory biomarker that would demonstrate the attributes of an 'ideal biomarker' for use in assessing IBD continues.

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