Emerging concepts in non-invasive monitoring of Crohn's disease

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Abstract: Inflammatory bowel disease (IBD) is an umbrella term for Crohn's disease (CD) and ulcerative colitis (UC). In light of evolving epidemiology of CD, its clinical management is still complex and remains a challenge for contemporary physicians. With the advent of new diagnostic and treatment paradigms, there is a growing need for new biomarkers to guide decision-making, differential diagnosis, disease activity monitoring, as well as prognosis. However, both clinical and endoscopic scoring systems, widely utilized for disease monitoring and prognosis, have drawbacks and limitations. In recent years, biochemical peptides have become available for IBD monitoring and more frequently used as surrogate markers of gut inflammation. Emerging concepts that revolve around molecular, stem cell, epigenetic, microbial or metabolomic pathways associated with vascular and epithelial gut barrier could lead to development of new CD biomarkers. Measurement of cell-derived microvesicles (MVs) in the blood of IBD patients is another emerging concept helpful in future disease management. In this review, we discuss novel concepts of non-invasive biomarkers, which may become useful in monitoring of CD activity and prognosis. We discuss metabolomics as a new powerful tool for clinicians to guide differential IBD diagnosis. In the coming years, new developments of prognostic tools are expected, aiming for breakthroughs in the management of patients with CD.

Keywords: Crohn's disease, inflammatory bowel disease, microbiota, gut barrier, mycobiota, stem cells, biomarkers, disease monitoring, microbiome, intestinal permeability

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Introduction

A number of different tests may be required to diagnose Crohn's disease (CD). Currently endoscopy-based evaluation is the most important diagnostic tool. With an increasing number of patients with clinical symptoms mimicking CD, the detailed diagnostic evaluation is critical to proper management and prognosis in patient follow up. The diagnosis of CD still relies on clinical experience and diagnostic tools such as microbiological and biochemical testing. Advanced gastrointestinal imaging, that is, computed tomography (CT) and magnetic resonance (MRI) are also invaluable diagnostic tools. In the years to come, we envisage a single surrogate biomarker as impossible to replace endoscopic mucosal assessment in the diagnosis of CD.

In contrast, the new biomarkers could be utilized in CD monitoring and could guide physicians in differential diagnosis of inflammatory bowel disease (IBD). In this review, we focus on emerging biomarkers that may become useful in the differential diagnosis of IBD and disease monitoring. The genetic and serologic immune markers currently lack clinical accuracy, and their detailed description is beyond the scope of this review, as has been discussed elsewhere.[1] Novel diagnostic research tools, for example, metabolomics, are also briefly discussed.

Traditional management of CD has been closely related to monitoring of clinical symptoms [e.g. using the Crohn's Disease Activity Index (CDAI), the Harvey-Bradshaw Index (HBI) or the Inflammatory Bowel Disease Questionnaire (IBDQ)], supplemented by endoscopic assessment tools [e.g. the Crohn's Disease Endoscopic Index of Severity (CDEIS), the Simple Endoscopic Score for Crohn's Disease (SES-CD) or the
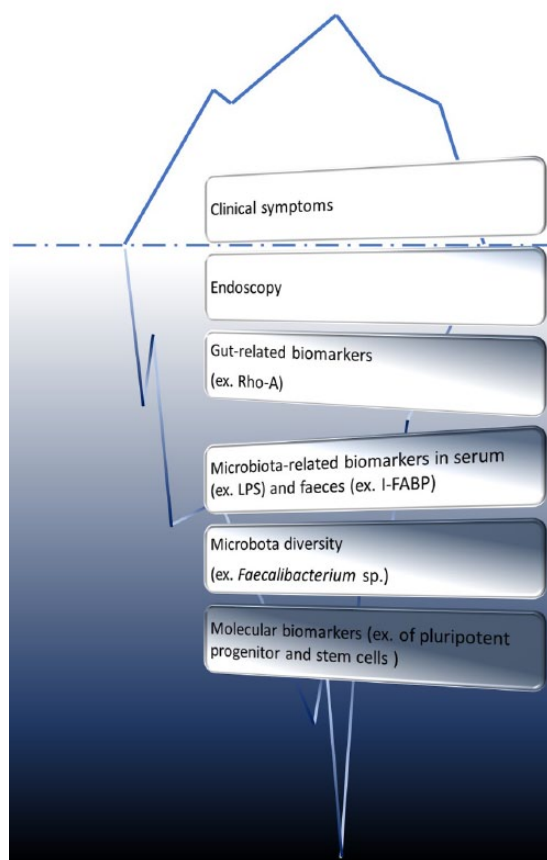
Figure 1. Current and emerging concepts in inflammatory bowel disease management.

Rutgeerts Score (RS)] for postoperative recurrence to assess deep mucosal healing, and the recently developed Lehman's score. However, these disease-activity-monitoring tools have several drawbacks and limitations. For instance, clinical scores are mostly subjective, and symptoms of irritable bowel syndrome (IBS) may confound the report of IBD patients without active bowel inflammation. Furthermore, endoscopic scores are poorly reproducible, highly dependent on the endoscopist's experience and are of course based on invasive procedures. Therefore, all of the visual scoring systems available in clinical practice so far have limitations.[2]

Recently, biochemical markers (e.g. calprotectin, lactoferrin) have become widely available, thus allowing for better objective monitoring of intestinal inflammation.[3] Although the aforementioned modalities are widely utilized in various combinations for disease prognosis, they cover only the tip of the iceberg of prognostic potential (Figure 1). With the advent of novel '–omics' non-invasive

technologies, clinicians await their practical validation and relevant correlations. Certainly, all these novel modalities should allow us to immerse in the unexplored waters around the bottom of the iceberg of 'deep remission'. However, the boundaries of this relatively new term[4] are not known. New definitions revolve around terms such as 'molecular', 'epigenetic', 'microbial' or 'metabolomic mucosal healing', to name a few.

The emerging field of microbiota research opens up new avenues for novel biomarker development in CD. Moreover, current research could fulfill this unmet need in CD management. Microbiota-based biomarkers may become useful in the differential diagnosis, as well as in monitoring and prognostication of CD. However, the field is still in its infancy and in need of continuous development from a clinical point of view.

Microbiota as a source of putative biomarkers in Crohn's Disease

The human intestine is colonized by multiple microorganisms including bacteria, archaea, viruses, and eukaryotes; namely, gut microbiota.[5] This microecological niche is dominated by the *Firmicutes* and *Bacteroidetes* phyla, followed by *Proteobacteria* and *Actinobacteria* and is responsible for multiple processes within the human body, such as decomposing undigested food particles to produce energy for colonocytes, synthesizing vitamins and amino acids, and increasing the bioavailability of the mineral components and lipid digestion.[6–8]

Most importantly, microorganisms participate in the rapid elimination of antigens present in the intestinal lumen. Mucin synthesis protects endothelium from pathogens and toxin invasion, consequently preventing their translocation into the bloodstream and the development of systemic inflammation. By means of competitive inhibition and in the presence of antimicrobial metabolic products (bacteriocins, hydrogen ions), microbiota limits the possibility of gut colonization by pathogens.[9] This process is done on a molecular level, by attaching pathogen-associated molecular patterns (PAMPs) to TLRs (Toll-like receptors), nucleotide-binding oligomerization domain-2 (NOD2) and Rig-I-like receptors (RLRs).[10]

Proper mechanisms of interaction between microbiota and gut-associated lymphoid tissue (GALT) affect tolerance to commensal bacteria and food

antigens.[11] Since the role of innate immunity in CD pathogenesis has already been established,[12,13] it seems that the disease is propagated by a dys-regulated response to the symbiotic microbiota and consequently defect in intestinal barrier integrity.[14] As a reliable predictor of the disease is still lacking, it seems that microbiota-associated bio-markers being involved in the onset of inflammation and dysbiosis that is followed by relapse may be a crucial diagnostic and therapeutic target.

The clinical relevance of microbial biodiversity in Crohn's disease

Intestinal microbial imbalance and a reduction in bacterial biodiversity, referred to as dysbiosis, has been well documented in CD.[15–18] In a pioneering study by Swidsinski and colleagues,[19] the abundance of mucosal microbiota in IBD patients was found to be positively correlated with its clinical course. The expression of numerous genes were lowered in the faecal samples of IBD patients compared with healthy volunteers.[20] By means of culture-independent molecular techniques, it was elegantly shown that bacterial α-diversity in CD patients was lowered, and abundance of several other groups of microorganisms elevated in comparison with healthy controls (HC)[21–23] and other clinical conditions; however, the results of different studies are conflicting and may be due to the small number of cases.

Importantly, *Faecalibacterium prausnitzii* has been associated with an increased risk of postresection recurrence of ileal CD.[24] Moreover, Pascal and colleagues[25] discovered that other butyrate producers, that is, *Christensenellaceae*, *Methanobrevibacter* and *Oscillospira* were decreased in CD. Similar observations were made by Takahashi and colleagues;[26] the researchers found that *Blautia faecis, Roseburia inulinivorans, Ruminococcus torques, Clostridium lavalense, Bacteroides uniformis*, all butyrate producers, were reduced in patients with CD. He and colleagues[27] demonstrated that CD microbiota are clustered in two different metacommunities: (a) an increase in bacterial producers of proinflammatory hexa-acylated lipopolysaccharides; and (b) a reduction in the community able to synthesize short chain fatty acids (SCFA). In another study, the *Fusobacterium* level was significantly decreased in CD.[25] On the other hand, Enterobacteriales, predominantly adherent-invasive *Escherichia coli* responsible for induction of proinflammatory cytokine [i.e. tumour necrosis factor (TNF)][25,28] synthesis were found to be increased in CD, with

lesions in the ileum,[29,30] as well as *Campylobacter concisu*s preferentially colonizing the gut of patients with CD.[31–33] Similarly, mucolytic bacteria such as *Ruminococcus gnavus* and *Ruminococcus torques* were discovered in abundance in CD patients.[34] Other studies delivered novel evidence of relative abundance of *Faecalibacteria* as a potential biomarker with high specificity in CD patients. In one of the most recent systematic reviews, Zhou and colleagues proved that lower levels of *Bacteroides* in the gut were associated with IBD in comparison with those in remission.[35]

Lower level of *Bacteroides* in the gut microbiota is associated with inflammatory bowel disease: a meta-analysis

Recently it was demonstrated that microbial community composition was more dysbiotic at the taxonomic level in CD patients. Six-bacterial profile abundance, that is, *Faecalibacterium, Peptostreptococcaceae, Anaerostipes, Christensenellaceae, Collinsella* and *Methanobrevibacter* were measured and found significantly decreased in CD patients.[25] Conversely, *Escherichia* and *Fusobacterium* richness were more pronounced when compared with HC and UC patients. Among *Firmicutes* and *Bacteroidetes* phyla, butyrate producers *Faecalibacterium prausnitzii* and *Bacteroides fragilis* respectively were previously found to be lowered in CD, species with protective effects within the gut microbiota composition.[24,36] Butyrate seems to be critical for the homeostasis within the gut. It serves as an energy source for the host and is involved in lipogenesis, gluconeogenesis, epigenetic processes, as well as suppression of inflammatory mediators. It was found that *Faecalibacterium* species suppressed experimental colitis *via* lowering nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation and inflammatory cytokines synthesis and by stimulating interleukin IL10 production.[24]

Overall, fewer bacteria with anti-inflammatory properties and more bacteria with proinflammatory properties compared with healthy subjects have been identified.[30,37,38] Members of the *Bacteroidetes* and *Firmicutes* phyla were found to be reduced in CD; conversely, abundance of *Enterobacteria*, mostly *Escherichia coli*, was observed. Prosberg and colleagues,[39] in their systematic review and meta-analysis (5 studies; 231 patients), found lower abundance of *Clostridium leptum, Faecalibacterium prausnitzii*, and *Bifidobacterium* in

patients with active CD. Zhou and Zhi,[35] in their meta-analysis, included 346 patients with CD, and revealed that level of *Bacteroides* in patients with active CD was lower than in the HC group. *Bacteroides* abundance was also fewer in CD patients in the remission phase than in the control group, as well as in the active phase of disease than in remission. However, obtained data were heterogenic due to different analytical methods (real-time quantitative polymerase chain reaction, fluorescence *in situ* hybridization, conventional culture), small sample size and ethnic differences.

Mycobiota as a potential source for novel non-invasive biomarkers in Crohn's disease

Apart from bacterial imbalance, mycobiota diversity was found altered in CD. It was proposed that the microenvironment of CD is favourably enriched by fungi rather than bacteria. Namely, the *Basidiomycota:Ascomycota* ratio and the *Candida albicans* count were found to be increased, while *Saccharomyces cerevisiae* abundance decreased.[18,40,41] Other species' numbers found elevated in CD included *Gibberella moniliformis, Alternaria brassicicola* and *Cryptococcus neoformans*. It is of interest that fungal microbiota correlated with the CD activity index and the degree of inflammation expressed by C-reactive protein (CRP) concentration.[42]

As dysbiotic therapy was found to be effective toward the bacterial component, mycobiota abundance was expanded, highlighting that fungal composition could be further researched as a causative agent.[43] Importantly, from a mechanistic point of view, the antifungal inflammatory response is known to be under the control of multiple IBD risk genes.[41] Moreover, ileal mucosa samples and gut lavages from CD paediatric patients were found to be enriched in *Caudovirales*, negatively correlated with bacterial diversity,[44,45] proving that the virome of CD should be considered when searching for a microbiota-associated biomarker.

The differential diagnosis of Crohn's disease: the role of metabolomics

Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind, the study of their small molecule metabolic profiles. It can give an instantaneous snapshot of the metabolic state of a cell or an organism. It uses specific technology to capture the metabolic processes. Widely used are nuclear magnetic resonance spectroscopy (NMR-S) gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS).

In CD, there have been a number of studies using these analytical techniques to identify metabolic fingerprints that could potentially be used as biomarkers to either diagnose CD or monitor disease activity. As with humans, it is not practical to study processes either on a cellular level or *in vivo* on the whole body. Different biological media have been studied and the results have been extrapolated to assess metabolic functions at a cellular level. Media studied include serum and plasma, urine, faecal extract and breath.[46] We will look into those four media in more detail.

Multiple clinical biomarkers of CD status have been studied so far, some of them commercially available. However, the most prevalent remain serum CRP and faecal calprotectin (FCP) concentrations.[47] Although a number of studies aimed to assess the utility of these biomarkers and to evaluate the correlation with endoscopic scores, relapse and postoperative recurrence prediction, their main value is in managing patients with CD, as reported by Mosli and colleagues.[48] Therefore, looking for a novel, accurate biomarker is a research priority.

A recent study showed that a serum profile including lipoproteins especially high-density lipoprotein cholesterol, choline, N-acetylglycoprotein and amino acids could differentiate between CD and UC but also between IBD and HC.[49] Another study showed that a plasma amino acid profile including histidine and tryptophan could differentiate CD patients from HC.[50] Furthermore, a study using GC-MS was able to separate CD from UC and controls. Metabolites included angiotensin IV, diphthamide gangliosides and combined kynurenine metabolites.[51] Finally, a small study using NMR-S showed a different lipidomic profile in CD patients compared with HC.[52] A study by Mortensten and colleagues[53] delivered evidence that serum extracellular matrix (ECM) components, namely degraded biglycan and citrullinated and matrix metalloprotease degraded vimentin (VICM) could serve as a biomarkers differentiating CD from UC. Also, patients with CD who are positive for anti-flagellin IgG antibodies: anti-CBir1,

anti-A4-Fla2, anti-Fla-X and anti-outer-membrane porin C antibody (anti-OmpC IgA) at baseline (perioperatively), all serological biomarkers directed against gut microbial antigens, were found to be at high risk of disease recurrence at 18 months in comparison with those negative for these markers.[54] As far as serum antibodies are concerned, antiglycan, antiglycoprotein 2 (anti-GP2) antigranulocyte-macrophage-colony-stimulating factor (anti-GM-CSF) antibodies in IBD attracted attention, as all these biomarkers were found to be associated with CD pathogenesis and linked to complicated phenotypes of the disease.[55] Also, as CD genetic background was established, small noncoding ribonucleic acids (miRNA), responsible for gene expression regulation and previously found to be associated with the immune disorder pathogenesis,[56] serum mi-R223 expression was shown as elevated in CD compared with HC and positively correlated to the disease activity [CDAI; erythrocyte sedimentation rate (ESR); simple endoscopic score for CD (SES-CD)].[57] Other markers, for instance serum platelet factor 4, are promising and reliable as biomarkers for IBD.[58]

Investigators of studies using urine were able to differentiate between CD and UC, and HCs. Levels of hippurate are found consistently low in CD patients.[59–61] The study by Alonso and colleagues[59] also demonstrated low levels of citrate, hydroxyisovalerate and dimethylglycine. A recent larger study which combined different IBD phenotypes showed statistically significant lower levels of acyl carnitine 4-α-hydroxyphenyl pyruvate in patients with CD phenotype compared with UC phenotype.[62]

Two recent studies have used faecal extracts from patients with CD and produced some interesting results. One study showed significant differences in patients with CD compared with UC in aspartic acid and glutamate concentrations; branch chain amino acids lysine, alanine, phenylalanine and butyrate were also different when CD patients and controls were compared.[63] The other examined IBD patients and their families and showed that siblings of IBD patients can have a different metabotype and different bowel microbial species to healthy controls or IBD patients.[64] Other faecal IBD biomarkers, including S100A12 (calgranulin), high-mobility group box 1, neopterin, polymorphonuclear neutrophil elastase, human neutrophil peptides, neutrophil gelatinase-associated lipocalin, chitinase 3-like-1 and others are discussed in detail elsewhere.[65]

Some studies have used more than one biofluid. Studies using both serum and urine were able to discriminate between CD and HCs but less so between CD and UC. Metabolites of interest included isoleucine, acetoacetate, hippurate, taurine, succinate, glycine, alanine and formate.[66,67] One study using terminal ileal (TI) biopsies and macrophages from affected individuals found differences in the lipidomic profile between CD patients and HCs.[68]

So far, all studies conducted showed promising results but most of them were individual efforts that looked at a specific metabolic activity. Coordinated studies looking at the most promising data and building on them should be the next step.

Gut barrier and intestinal permeability: novel targets for Crohn's disease monitoring?

Alterations in structure and function of the gut barrier play a significant role in the pathogenesis of IBD. Kiesslich and colleagues[69] in their elegant study utilizing confocal endomicroscopy documented how cell shedding and barrier loss predicted the relapse of IBD, and had the potential to serve as a diagnostic tool for the management of the disease. Chang and colleagues[70] observed how IBD patients frequently suffer from ongoing bowel symptoms of diarrhoea and abdominal pain despite mucosal healing and investigated whether impaired intestinal permeability contributed to these symptoms. The authors found that increased gut permeability correlated with increased severity of bowel symptoms beyond mucosal healing and drew the conclusion that resolution of mucosal permeability might improve outcomes of patients with IBD.[70] It is worth noting that tests measuring intestinal permeability have long been used in medical practice[71,72] (Table 1), however their clinical applicability was rather low. Endoscopic techniques, while yielding promising results, are invasive, timely, expensive and require experienced endoscopists. Microbiome research presents new views on the role of microbiota in shaping gut barrier; in parallel, new solutions for novel biomarkers emerge.

Dysbiosis is known to induce changes in intestinal barrier permeability.[73] Intestinal barrier is the physical structure present in the intestinal tract (Figure 2). The most important element of the barrier is the epithelium, primarily made up of

Table 1. Approaches for measuring intestinal permeability.

Approach type	Test name	Specificity to location	Sample	Notes
Functional	Ussing chamber	Site specific	Biopsy	Invasive, *ex vivo* test
	Lactulose/mannitol	Small bowel	Urine	Time consuming
	Sucrose/glucose*	Stomach	Urine	Time consuming
	Sucralose*	Colon	Urine	Time consuming
	PEG 4000/400	Whole gut	Urine	Time consuming
	^{51}Cr-EDTA	Whole gut	Urine	Radioactive
Bacteria related	LAL-assay	Whole gut	Plasma	
	EndoCAb	Whole gut	Serum	Acute-phase specificity
Inflammation-related	Calprotectin	Whole gut	Faeces	Unspecificity
	α1-anti trypsin	Small bowel	Serum	
	sIgA	Whole gut	Serum	Low specificity
	zonulin	Colon	Faeces	
Epithelial damage	Citrulline	Small bowel	Plasma	
	I-FABP	Site specific	Plasma	Acute-phase specificity
Histological biomarkers	Tight junction expression**	Site specific	Biopsy	Invasive, *in vivo* test
	Defensins expression**	Site specific	Biopsy	Invasive, *in vivo* test
	Loss of Paneth cells	Site specific	Biopsy	Invasive, *in vivo* test
	Shedding of epithelium	Site specific	Biopsy	Invasive, *in vivo* test

*In combination with lactulose/mannitol test; **mRNA and protein level.
PEG, polyethylene glycol; 51Cr-EDTA, chromium labelled ethylenediaminetetraacetic acid; LAL, lymulus amebocyte lysate assay; EndoCAb, endogenous endotoxin-core antibody; sIgA, secretory immunoglobulin A; I-FABP, intestinal fatty-acid-binding protein; mRNA, messenger ribonucleic acid.

enterocytes (80%) responsible for nutrient absorption and stimulating immune activity by secretion of cytokines and expression of immune receptors in the cell membrane. Integrity of the intestinal barrier is provided predominantly by close interlinking between intestinal epithelial cells (IECs), namely tight junctions (TJs). These are protein complexes composed of claudins, occludins, junctional adhesion molecules (JAMs) and tricellulins, which contact the cytosol with *Zonula occludens* (ZO) proteins, being conjugated to the cytoskeleton of the IECs. As a result of the phosphorylation of myosin light chains actin contraction occurs which relaxes the structure of the TJs and allows the luminal content to be transported paracellularly. Structural and functional homoeostasis of this structure limits the passage of pathogenic microorganisms and other harmful antigens to the blood.[74]

Elevated intestinal permeability was established as well in quiescent IBD as first-degree relatives of CD patients.[75–82] Transepithelial electrical resistance was altered in mild and moderately
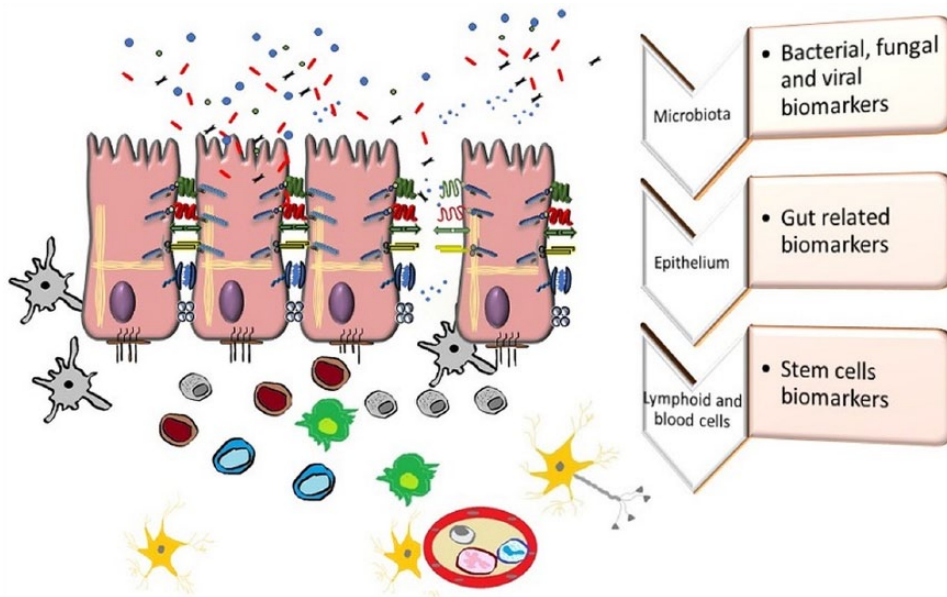
Figure 2. Gut-related biomarkers in inflammatory bowel disease.

active CD.[83] Other studies proved that increased small bowel permeability to sugar molecules or ^{51}Cr-EDTA.[75] Moreover, studies state that in CD patients, hyperresponsiveness toward dysbiotic agents such as nonsteroidal anti-inflammatory drugs (NSAIDs) occurs as it was demonstrated that intestinal permeability is elevated even in patients with non-inflamed mucosa[84,85] that in turn may proceed to relapse for small intestinal CD[86] in humans and intestinal inflammation in animals.[87]

Of note, CD patients and their relatives excrete more FCP, positively correlated with endoscopic activity and neutrophil migration towards the intestine[88,89] in comparison with HC,[90] although this biomarker is not specific to IBD. A widely studied inflammation-related biomarker called zonulin was found to be elevated in both faeces and serum during the active CD phase, but not in UC.[91] Another barrier-integrity-specific molecule is Rho-A, which was found to be downregulated in IBD[92] and Rho-associated kinase upregulated in inflamed mucosa of CD patients.[93] It was proved that these alterations are key mediators of chronic inflammation, cytoskeleton rearrangement and skewed cell shedding.

Since TJs are thought to be critical for intestinal barrier integrity, it was shown that in CD patients, TJs form particle-type structures with no

continuity, and in the active stage of disease, the expression of claudin 3, 5, 8 and occludin was found to be decreased.[94–96] Conversely, claudin-2 abundance was proved to be increased during inflammation that in turn may lead to leak flux diarrhoea. In parallel to claudin-2 overexpression and decreased production of occludin, increased synthesis of proinflammatory molecules may initiate immune response or maintain inflammation within the intestinal wall.[97] The new CD biomarkers associated with gut barrier alterations are listed in Table 2.

Measuring the unmeasurable: dysbiosis as a target in Crohn's disease monitoring?

There is mounting evidence that microbiota composition in CD is altered, although the dysbiosis may be both causative and a consequence of inflammation. In paediatric cohorts,[15,41,43] the researchers found stool and mucosa dysbiosis in newly diagnosed and nontreated children, proving that microbial imbalance reflects the ongoing inflammation but may be triggered by certain environmental factors. Of note, there is also a body of evidence that microbial signature may be clinically relevant in management of CD (Table 3). On the other hand in cross-sectional studies, microbial composition differed even between IBD twins, suggesting that certain dysbiosis may be instead associated with the disease

Table 2. Gut-related biomarkers in Crohn's disease.

Biomarker	Relevance	Reference
Microbiota-related biomarkers		
Adherent-invasive *Escherichia coli* (AIEC)	A greater pathogenic effect of AIEC (elevated TNF-α production linked to granuloma formation and lower expression of miRNA, thus decreased autophagia) typically found in chronic lesions in intestine and always present in early postoperative-recurrence lesions	Bosca-Watts *et al.*;[98] Barnich and Darfeuille-Michaud;[99] Nguyen *et al.*[100]
Faecalibacterium sp.	The abundance is decreased in patients with CD and may predict postresection relapse; the bacteria count was found to be reduced in patients with active CD in comparison with the disease in remission	Sokol *et al.*;[24] Pascal *et al.*;[25] Prosberg *et al.*[39]
Enterococcus faecalis	Decreased abundance related to clinically active CD; more richness in stool associated with ileal rather than ileocolonic CD	Pascal *et al.*;[25] Zhou *et al.*[101]
Bacteroides sp.	CD microbial ecosystem was found to be significantly lowered in *Bacteroides* sp., both in active and remission patients in comparison with HCs; the abundance was even lower in remission patients as compared with HCs and active phase of the disease	Zhou and Zhi[35]
Clostridium leptum	Lower abundance of the species in patients with active CD in comparison with remission phase was found	Prosberg *et al.*[39]
Streptococcus sp.	Percentage of the species positively correlated with the postoperative recurrence development compared with patients who stayed in remission	Pascal *et al.*[25]
Anaerostipes sp.	The microbiota of CD patients is almost absent in these species when compared with HCs and UC	Pascal *et al.*[25]
Methanobrevibacter sp.	Almost no abundance in CD in comparison with HCs and UC	Pascal *et al.*[25]
Collinsella sp.	High abundance in CD in comparison with almost absent in HCs and UC	Pascal *et al.*[25]
Fusobacterium sp.	High percentage found typically in UC, not in CD	Pascal *et al.*[25]
Basidiomycota/Ascomycota ratio	The ratio was high in active CD and normal in remission	Sokol *et al.*[18]
Saccharomyces cerevisiae	Antibodies against *Saccharomyces cerevisiae* mannan detected in CD patients; abundance of *Saccharomyces cerevisiae* decreased in CD	Sokol *et al.*;[18] Main *et al.*[102]
Candida albicans	The abundance significantly increased in CD flare compared with disease in remission	Sokol *et al.*[18]
Short chain fatty acids (SCFAs)	CD bacterial dysbiosis reduces the community (*Bifidobacterium* species, *Faecalibacterium prausnitzii*, *Alistipes shahii*, and *Roseburia* species) able to produce SCFAs	He *et al.*[27]
Gut barrier-related biomarkers		
Lipopolysaccharide (LPS)	Even sixfold increase in serum LPS concentration in CD; the concentration positively correlated with disease activity	Magro *et al.*[103]
Alpha-1-antitripsin (A-1AT)	A faecal biomarker associated with clinical relapse in patients with CD of the distal ileum; replaced with CP	Biancone *et al.*[104]

Table 2. (Continued)

Biomarker	Relevance	Reference
Calprotectin (CP)	Positively correlated with endoscopic activity and neutrophil migration towards the intestine	Burri and Beglinger;[88] Kopylov *et al.*;[105] Egea *et al.*;[106] Sipponen *et al.*;[107] Bar-Gil Shitrit *et al.*[108]
Zonulin	Faecal and serum concentration positively correlated with activity of the disease	Malíčková *et al.*[91]
Intestinal fatty acid binding protein (I-FABP)	Serum I-FABP concentration is increased parallel to the CD activity index	Sarikaya *et al.*[109]
Claudins	Claudin 3, 5, 8 and 2 expression was found to be decreased and increased respectively in the active CD stage	Zeissig *et al.*[94]
Occludin	Decreased expression during active phase of CD	Al-Sadi *et al.*;[95] Buschmann *et al.*[96]
Other biomarkers		
Serum		
Cathelidicin (LL37)	Inverse correlation to clinical CD activity	Tran *et al.*[110]
CD26 (dipeptidyl peptidase IV)	Serum CD26 level lowered in both active and inactive CD when compared to controls	Magro *et al.*[103]
Faeces		
B cell-activating factor (BAFF)	Positively correlated with endoscopic inflammation in CD patients	Fu *et al.*[111]
Lactoferrin	Negatively correlated with CD severity; paediatric CD patients with positive result of faecal occult blood test had higher concentrations of lactoferrin than the individuals with negative result of this test; however, available data show conflicting results	Sipponen *et al.*;[107] Boon *et al.*;[112] Borkowska *et al.*;[113] Sidhu *et al.*[114]

CD, Crohn's disease; HC, healthy control; TNF-α, tumour necrosis factor-alpha; UC, ulcerative colitis.

phase.[21,115] Of note, inflammation being an oxidative state induces the synthesis of particular electron acceptors for facultative anaerobes, promoting the enrichment of gut niche in pathobionts.[116] Consequently, dysbiosis leads to an altered metabolic profile. Morgan and colleagues[117] found that many more metabolic pathways were different between IBD and HCs than genetic clades, proving that functional alterations should be of particular interest in dysbiotic studies. It is notably important as different metabolite syntheses might induce host IBD risk factors, as seen in bile acid signalling studies[118–120] or lowered short chain fatty acids involved in immune tolerance, thus inflammation development.[121] In conclusion, multiple studies highlighting certain microbiota alteration as pathogenetic factors should be taken with caution, as little prospective data exist. Different '-omic' approaches may serve as a development area within mucosal homeostasis and inflammation, although not widely used in clinical practice.

Pluripotent progenitor and stem cells as surrogate prognostic markers of mucosal healing and clinical remission in Crohn's disease

While stem cell therapy in Crohn's disease has met significant scientific and clinical attention,[126,127] the usefulness of stem cells as biomarkers in IBD has been less studied. Developmentally early cells,

Table 3. Microbial biomarkers and their clinical relevance in management of Crohn's disease.

Microbial biomarker	Clinical relevance	Reference
Faecalibacterium prausnitzii, Clostridium coccoides	Reduced abundance in postoperative endoscopic recurrence	Sokol *et al.*[24]
F. prausnitzii, C. coccoides, Clostridium leptum, Bacteroides sp.	Reduced abundance as a predictor of endoscopic recurrence following infliximab withdrawal	Rajca *et al.*[122]
F. prausnitzii, Nitrosomonas sp., *Bifidobacterium* sp., *Desulfotomaculum thermobenzoicum, Ruminococcus flavefaciens, Desulvibrio vulgaris*	Greater abundance in patients experiencing remission after surgery	de Cruz *et al.*[123]
Enterococcus faecalis, unknown species of *Erysipelotrichaceae*	Greater abundance in patients with CD disease localised in the ileum than in the ileocolon	Pascal *et al.*[25]
Bacteroides sp. (*Bacteroides plebeius*), *Dorea* sp. (*Dorea longicatena*), *Ruminococcus* sp., *Dialister* sp.	Increased abundance in postoperative remission patients	Mondot *et al.*[124]
Gemmiger formicilis, Enterococcus durans, Ruminococcus lactaris	Increased abundance in postoperative recurrence patients	Mondot *et al.*[124]
Desulvibrio longus, Streptococcus pneumoniae	Greater abundance in patients with postoperative recurrence	de Cruz *et al.*[123]
Lachnospiraceae, Erysipelotrichaceae, unidentified genus within *Clostridia*, all members of the phylum *Firmicutes*	Reduced abundance in postoperative endoscopic recurrence	Dey *et al.*[125]
Rhodobacteraceae (of class *Alphaproteobacteria*), an unknown *Proteobacteria, Rhizobium* sp. (also *Alphaproteobacteria*)	Greater abundance associated with postoperative recurrence	Dey *et al.*[125]

including hematopoietic stem progenitor cells (HSPCs), mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and very small embryonic-like stem cells (VSELs), are mobilized in peripheral blood (PB) in response to tissue/organ injury.[128,129] The evidence is mounting that cells expressing markers for MSCs, EPCs, and small Oct-4+ Nanog+ SSEA-4+ CXCR4+ Lin⁻ CD45⁻ VSELs are mobilized into PB in patients with CD.[130] The mobilized cells express genes at the messenger RNA (mRNA) level, playing a role in development and regeneration of gastrointestinal epithelium.[131–133] Marlicz and colleagues reported an increase in the number of circulating MSCs, EPCs and small primitive cells expressing the VSEL phenotype in IBD patients with active disease.[130] The most significant increase was observed for CD105+/STRO-1+/CD45⁻ cells enriched for MSCs. The number of circulating CD34+/KDR+/CD31+/CD45⁻ EPCs also increased significantly. In subpopulation of cells enriched for VSELs, cells stained positive for CXCR4, CD34, CD133 and negative for lineage markers and CD45 antigen were elevated in PB of patients with CD. Of clinical importance, the highest numbers of VSELs were observed in younger patients with active ileocaecal mucosal lesions in the colon.[130]

Furthermore, the authors observed that hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) correlated positively with advancement of mucosal lesions in the colon.[134] Active mucosal lesions were associated with higher

plasma levels of HGF and VEGF in patients achieving clinical remission. Active CD was associated with strong mRNA upregulation of intestinal early differentiation markers (Lgr-5, ASCL-2, Msl-1, Bml-1, Dclk-1) in circulating PB mononuclear cells. Moreover, higher plasma C5a levels in IBD in comparison with controls were noted. The study also revealed that the number of VSELs mobilized in PB in patients remaining in clinical remission (not longer than 6 weeks since flare up) was significantly increased compared with HC.[134] The authors observed that the number of CXCR4+, Lin⁻, CD45⁻ cells mobilized in PB in patients in clinical remission was associated with active mucosal lesions in the colon. The significance of multifold upregulation of Lgr-5+ mRNA (marker of intestinal stem cells) in peripheral bone-marrow-derived mononuclear cells (BMMNCs) of Crohn's patients with active disease awaits scientific explanation.

Evidence is mounting for endothelial impairment contributing to the onset, progression and recovery from inflammatory injury.[135,136] Of particular interest, EPCs, derived from bone marrow hematopoietic stem cells, have the capability to migrate to the site of endothelial damage through the peripheral circulation.[137] Boltin and colleagues[137] studied patients with CD in varying stages of disease activity assessed using the CDAI. Enrolled patients were being treated either with biological therapy (infliximab) or immunomodulators. The evaluation of circulating EPCs employed antibodies to the biomarkers of cluster differentiation (CD34), VEGF receptor-2, CD133, and CD45. Researchers reported a significant increase in the percentage of EPCs of the peripheral mononuclear cells in patients with CD as compared with healthy individuals. Of prognostic significance, the authors did not find the association between EPC percentages and other factors such as age, sex, CDAI, disease duration, duration of biological therapy or habits (e.g. smoking). Perhaps more advanced techniques such as tissue microarrays or RNA sequencing are needed in order to unravel such associations.[136] The current studies are seeds for the field, where enumeration of stem/progenitor cells in PB could serve as a surrogate parameter in accessing treatment efficacy in IBD. At this point, it is important to note that mobilization and activity of stem and progenitor cells could be influenced by numerous factors, such as drugs (e.g. antibiotics),[138] proton-pump inhibitors,[139,140] infection,[141,142] physical activity,[143–146] and age.[147]

Therefore, various endogenous and environmental factors in patients with CD should be evaluated before assessing any risk factors based on biomarker measures. Moreover, intestinal microbiome could be a source of various molecules interacting with progenitor/stem cells affecting their potency. Conversely, innate immunity mechanisms could be triggered as a response to various microbial communities in the gut, for example, bone marrow expressed antimicrobial cationic-peptide-LL-37-enhanced responsiveness of hematopoietic stem progenitor cells to an SDF-1 gradient and accelerated their engraftment after transplantation.[148] This observation is important in light of the work by Raftery and colleagues[149] who report that short-term treatment with 2000 IU/day vitamin D significantly increased 25 (OH) D levels in CD patients in remission and was associated with increased LL-37 concentrations and maintenance of intestinal permeability (IP) achieving $25(OH)D \geqslant 75$ ng/ml. This observation is clinically relevant, as active CD was associated with low serum 25-OH vitamin D and patients who smoked had lower 25-OH vitamin D levels than patients who did not smoke, independently of disease activity.[150,151]

Blood-derived microparticles and inflammatory bowel disease

Extracellular microvesicles (ExMVs) are part of the cell secretome. Evidence is mounting that ExMVs are involved in chronic autoimmune diseases, including IBD. Ratajczak and colleagues[152] demonstrated for the first time that ExMVs carry functional RNA species and proteins from one cell to another, an observation that paved up the new road to the new field of research of horizontal transfer of bioactive molecules in cell-to-cell communications.[153] This observation opened up the gates to novel concepts, in which the presence of mRNA, noncoding RNA, and miRNA in ExMVs in blood and other biological fluids gave the possibility of employing ExMVs as new biomarkers for disease conditions. Since then, ExMVs became a target for 'liquid biopsy' approaches. Tziatzios and colleagues found that circulating levels of platelet-derived microparticles (PDMPs) were increased in CD patients but did not correlate with disease activity. 5-ASA treatment was associated with lower levels of PDMPs, while anti-TNF-α treatment did not influence expression of ExMVs in IBD patients.[154] Similarly, circulating PDMPs and their counterparts in particular annexin (−) PDMPs were increased in IBD patients with active

disease. Annexin (+)/(−) ratio proved to be the most reliable distinctive PDMP index between healthy individuals and IBD patients.[155] Of interest, Leonetti and colleagues documented that ExMVs from CD patients altered endothelial and vascular function. The authors concluded that ExMVs may play a role in CD pathophysiology and vascular-dependent intestinal damage.[156] Of clinical significance, our team observed that CD133+ cells and CD133+ cell-derived MVs expressed mRNAs for several antiapoptotic and proangiogenic factors, including tyrosine-protein kinase receptor (Kit) ligand, insulin growth factor-1, vascular endothelial growth factor, basic fibroblast growth factor, and interleukin-8.

More importantly, the CD133+ cell-derived MVs chemoattracted endothelial cells and displayed proangiogenic activity in laboratory assays.[157] Further Ratajczak and colleagues presented the evidence that telocytes mediate several of their biological effects in various organs by releasing ExMVs enriched in mRNA, miRNA, proteins, and several biological mediators to the target cells.[152] Interestingly, Milia and colleagues delivered the evidence that in CD, the loss of telocytes might have important pathophysiological implications contributing to the architectural derangement of the intestinal wall and gut dysmotility.[158] Despite promising data, the field of ExMVs awaits clinical validation and practical application in the IBD clinical setting.

Targeting gut-liver axis to chase for new Crohn's disease biomarkers

Aside the aforementioned microbial or stem-cell-related markers available for future clinical application in CD, at least two other subjects deserve discussion – the topics of incidence and prevalence of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) among patients with IBD.[159] Younossi and colleagues,[160] in their meta-analysis (MA) that included more than eight billon individuals, found that one quarter of the adult world population might have been diagnosed with NAFLD/NASH. Of importance, gut–liver axis alterations have been implicated in the pathogenesis of NAFLD[161] and assessment of NASH severity could serve as a marker of intestinal inflammation.[162]

Reported risk factors of NASH in IBD include past small bowel surgery, obesity, use of steroids, and active disease with long duration. Moreover, a 'trend to significance' was found in this MA for anti-TNF-α medications as a risk factor for NAFLD and NASH.[159] However, the analysed data were biased and no clear conclusions could have been reached. The hypothesis of whether measuring the severity of NASH, either by means of invasive techniques (e.g. liver biopsy) or non-invasive techniques (e.g. fibroscan) could give us guidance towards severity of IBD remains to be tested.

Conclusion

New discoveries in the field of microbial and stem-cell-based biomarkers could bring new solutions to the management of patients with IBD.

Future studies could utilize small bowel endoscopic images (e.g. with capsule endoscopy) complemented by microbiological or cytologic fluid sampling of altered small bowel mucosa and further correlated with IBD progression.[69,163–165] *In vivo* molecular imaging of gut mucosa in IBD has prognostic potential as a personalized diagnostic tool.[166] Developing a simple wireless endoscopic capsule, capable of gathering confocal endomicroscopic images and sampling the intestinal fluid is not far away from realization. In fact, the intestinal gas capsule has entered the phase of human experimentation and results are awaited.[167] Similarly, volatile organic compounds in breath have been studied for some time now in patients with CD. None looked specifically at CD patients but rather looked at IBD as a cohort. The authors of two studies found significant differences in pentane, ethane and propane,[168,169] and of two others, claimed a unique breath fingerprint in patients with IBD.[170,171]

With the advent of novel '-omic' technologies, the development of new sensitive and specific biomarkers to monitor IBD is within the reach of scientists and clinicians. Despite the vast area of available data on IBD biomarkers, the ideas presented in this review offer new avenues for novel research and clinical trials in CD patients. As it is probably unlikely that a single biomarker will reliably monitor the disease, we envisage that contemporary clinicians enriched with an armamentarium of novel diagnostic and prognostic tools, would still need to rely on their experience to measure the faith of the disease. Extensive work in the field is expected to bring these new

tools into everyday medical practice. Currently, it is impossible to predict their accuracy, cost and ease of use. Future clinical trials with biomarkers should compare them with hard endpoints such as surgery, hospitalization, disease complication or endoscopic deep mucosal remission. Currently, lack of these comparisons in most of putative biomarkers' data is a general drawback of the field.

Conflict of interest statement
The authors declare that there is no conflict of interest.

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