



The Role of Gut Barrier Dysfunction and Microbiome Dysbiosis in Colorectal Cancer Development

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Accumulating evidence indicates that breakdown of the protective mucosal barrier of the gut plays a role in colorectal cancer (CRC) development. Inflammation and oxidative stress in the colonic epithelium are thought to be involved in colorectal carcinogenesis and the breakdown of the integrity of the colonic barrier may increase the exposure of colonocytes to toxins from the colonic milieu, enhancing inflammatory processes and release of Reactive Oxygen Species (ROS). The aetiological importance of the gut microbiome and its composition – influenced by consumption of processed meats, red meats and alcoholic drinks, smoking, physical inactivity, obesity - in CRC development is also increasingly being recognized. The gut microbiome has diverse roles, such as in nutrient metabolism and immune modulation. However, microbial encroachment towards the colonic epithelium may promote inflammation and oxidative stress and even translocation of species across the colonic lumen. Recent research suggests that factors that modify the above mechanisms, e.g., obesity and Western diet, also alter gut microbiota, degrade the integrity of the gut protective barrier, and expose colonocytes to toxins. However, it remains unclear how obesity, lifestyle and metabolic factors contribute to gut-barrier integrity, leading to metabolic disturbance, colonocyte damage, and potentially to CRC development. This review will discuss the interactive roles of gut-barrier dysfunction, microbiome dysbiosis, and exposure to endogenous toxins as another mechanism in CRC development, and how biomarkers of colonic mucosal barrier function may provide avenues for disease, prevention and detection.

Keywords: colorectal cancer, dysbiosis, biomarkers, colonic mucosal barrier, microbiome

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer related death and the third most commonly diagnosed in the world, with 1.8 million new cases in 2018 (1). Improvements in methods and screening programs, such as immunochemical faecal occult blood tests (FIT) and colonoscopy, have reduced mortality rates thanks to the detection and surgical removal of pre-cancerous colorectal adenomas (CRAs) or early-stage cancers (2). Unfortunately, despite increased screening strategies, disease diagnosis is still

more often at advanced stages and is associated with poorer prognosis. Accumulating evidence suggests that genetic susceptibility, environmental exposure, metabolic dysfunction, immune and inflammatory factors, microbiome composition and breakdown of gut barrier integrity play major roles in CRC aetiology (3). While most CRCs are considered sporadic, up to 35% is attributed to inherited susceptibility (4). High-penetrance germline mutations in mismatch DNA repair (*MMR*) genes and in *Wnt* (*APC*), *TGFbeta/BMP* signalling pathways (e.g., *MADH4*, *SMAD4*, *BMPRIA*) predispose to hereditary CRC syndromes (4). However, these mutations only account for about 6% of CRC while the inheritance of low-risk variants contribute a larger proportion of the genetic factors implicated in CRC development (5). Thus far, genome-wide association studies (GWAS) have identified around 100 loci associated with sporadic CRC, in or proximal to known CRC-related genes and pathways (e.g., *BMP2*, *BMP4*, *SMAD7*, *CCND2*, *GREM1*) and in genes not previously linked to CRC (*CDKN1A*, *EIF3H*, *TPD52L3*, *ITIH2*, *LAMA5*, and *LAMC1*) (6, 7). Modifiable lifestyle habits likely explain the worldwide heterogeneity in CRC incidence rates (5). It is well established that increased consumption of processed meats, red meats and alcoholic drinks, smoking, physical inactivity, obesity and adult attained height increase the risk of CRC development, while consumptions of fibres and calcium, and vitamin D levels are associated with a reduced risk (8). Furthermore, although age represents one of the risk factors for CRC, over the recent decades, the incidence rate for those under 50 years old has increased (9), underlying the relevant role of dietary and lifestyle environmental factors in CRC aetiology in addition to genetic predisposition (3).

Thus, the potential preventability of CRC depends on detailed understanding of its aetiology and interactive underlying mechanisms of development.

The role of the gut barrier is being increasingly recognized as pivotal to health and its dysregulation associated with a broad range of diseases, including celiac disease inflammatory bowel disease (IBD) and CRC (10–13). Metabolites derived from the diet can interact with the intestinal epithelium causing stimuli that may directly induce structural damage in epithelial cells and activate pro-carcinogenic signalling pathways, or by indirectly interacting with the gut microbiota (14).

Experimental and observational evidence suggests that the microbiome and its interactions with the human host are involved in most of the biological processes that regulate health and disease (15). The role of dietary and lifestyle factors in impacting microbiome composition has been widely discussed in the literature and it is known that they can affect the relationship between host and microbiome, causing alterations in gut barrier function and immune response (3, 16).

Recently, research has focused on whether disturbance in the balance of commensal microbial species may be carcinogenic and could provide a mechanistic link between dietary and lifestyle aetiological risk factors in CRC development (17). A direct role of the microbiota in carcinogenesis includes infection with Human Papilloma Virus (HPV), *Helicobacter pylori* (*H. pylori*) and Hepatitis B/C virus as the main aetiological risk factors for cervical, gastric and liver cancer development, respectively (18–20). While evidence

suggests that microbes in the colonic lumen have the capacity to be carcinogenic given access to colonocytes, they must circumvent barrier functions within and around the intestinal cells (3, 11). Based on the observation of the “leaky gut” in patients with intestinal diseases and the dysbiosis associated with the development of several chronic inflammatory disorders and CRC (21, 22), the study of the colonic barrier status and the markers associated with its damage may aid detection of early stages of intestinal disorders, including CRC. The examination of markers of colonic mucosal barrier dysfunction throughout the development of CRAs and CRCs may help to elucidate the dynamic interaction between the colonic epithelium and commensal bacteria in disease and health status.

In this review, we discuss the dynamic role of colonic mucosal barrier dysfunction (focusing on the mucus layer and epithelial cell lining components of the gut barrier), microbiome dysbiosis, and exposure to endogenous toxins as co-factors in CRC development. We also summarize the latest findings regarding the protein and metabolite markers of colonic mucosal barrier integrity and how these may provide new insights into CRC pathogenesis and as biomarkers of disease detection and prevention.

MICROBIOME DYSBIOSIS AND THE GUT EPITHELIAL BARRIER IN COLORECTAL CANCER DEVELOPMENT

Recent compelling evidence in both animal models and human studies suggest that infections by bacterial pathogens, commensal microbial dysregulation, and resultant alterations in microbial products are involved in CRC development (17, 23–25). Interactions of Western-type lifestyle habits (e.g., obesity, unhealthy diet), host genetics and immune responses are thought to induce microbiome changes, eventually leading to bacterial dysbiosis and shift the balance of metabolic function from beneficial to detrimental (17, 24).

The gut barrier is a dynamic and complex environment and acts as a physical and chemical barrier that suppresses the access of pathobionts, antigens and other invasive bacteria into the host (26). The outer mucus layer contains the commensal bacteria that produce antimicrobial proteins and secretory immunoglobulin A (IgA). In the lumen, bacteria and antigens are degraded by the action of gastric, pancreatic, and biliary juice while commensal bacteria produce bacteriocins, modify the pH of the luminal content, and compete for nutrients to inhibit the colonization of pathogens (14, 27). In the epithelium layer, epithelial cells contain tight junction complexes critical for sealing paracellular spaces between colonocytes, maintaining selective permeability and barrier integrity (28, 29). Furthermore, epithelial cells can transport the luminal content and produce antimicrobials to eliminate microorganisms and potentiate the action of monocytes and macrophages (27). The lamina propria contains cells of the immune and adaptive response able to secrete immunoglobulins and cytokines (27). The maintenance of a functional colonic mucosal barrier is essential to guarantee a healthy condition and the luminal confinement of bacteria is one of the key roles of the gut

mucosa. Over time, the protective capacity of the colonic mucosal barrier is affected allowing greater bacterial translocation and entry of toxic microbial products, such as pro-inflammatory endotoxins and bacterial metabolites, across the colonic epithelium (24, 30). These exposures may cause localized inflammation and the release of reactive oxygen species (ROS) within colorectal tissues, which may be causative or promotive of neoplastic processes. However, it remains unclear to what extent lifestyle factors impact these processes and whether these events are involved in CRC aetiology, or are consequences of the disease process (5, 30–33). Saus et al. define “driver” bacteria as those that conduct the colonic tissue damage, and “passengers” for those that merely profit from the microenvironment altered by the disease (34). Damage to the gut epithelial barrier mediated by microbes may promote carcinogenesis due to production of bacterial derived genotoxins, e.g., colibactin, and decreasing beneficial bacterial metabolites, such as short chain fatty acids (SCFA) (35, 36). SCFAs help to maintain gut barrier function and it has been shown that enhanced butyrate production may preserve the gastrointestinal epithelial lining through increasing expression of tight junction proteins (37). The breakdown of colonic barrier integrity and functionality impact colonic permeability facilitating microbial encroachment towards the colonic epithelium and greater exposure to toxic, mutagenic, and carcinogenic compounds from the colonic milieu, and bacterial translocation into the epithelium. Thus, there appears to be an intrinsically key relationship between the microbiome and the gut barrier in maintaining a healthy colorectal tract.

Although the definition is not well established and the composition of the microbiome varies among individuals (38), a healthy gut microbiome might be identified as a bacterial community residing in the gastrointestinal tract able to conserve defined functional genes in physiologic conditions (39). In the intestinal microbiome in the absence of evident disease, *Bacteroides* and *Firmicutes* represent a major part of the bacteria in the gut while phyla such as *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Cyanobacteria* and *Fusobacteria* are present in lower abundance (40). Any shift in gut microbial composition might reduce or inhibit the beneficial effects of the physiological functions of commensal microbes and impact the health/disease status of the host (41). Use of antibiotics from after one day of treatment can reduce microbiome diversity (42). The composition of the microbiome can be also impacted by host genetic variation such as in the *VDR* gene, encoding the vitamin D receptor (43) or the *NOD2*, *LCT* and *MUC2* genes, implicated in the regulation of immune response and secretion of anti-bacterial compounds (44), genetic regulation of *Bifidobacterium* abundance (45) and mucin secretion in the gastrointestinal tract (46). Variations in *FUT2*, a gene regulating the expression of histo-blood group antigens on the gastrointestinal mucosa (47), have been associated with differential interactions with intestinal bacteria and also with CRC risk (6). Furthermore, altered expression of DNA repair or immune-inflammatory pathways, such as innate immunity genes, including *MyD88*, *TLR4* and *TLR5*, have been associated with changes in the microbiome composition (48).

Several research groups have evaluated microbiome components as CRC screening biomarkers, employing qPCR of extracted DNA from either stool, including secondary use of stool based diagnostic

tests (e.g., FIT), or tumour tissue samples, to associate the relative abundance of bacterial species with colorectal lesions. Bacterial species most often observed to be over-represented in CRC from these studies include *Fusobacterium nucleatum* (*Fn*), *Enterococcus faecalis*, *Streptococcus gallolyticus* (*SGG*), enterotoxigenic toxin producing *Bacteroides fragilis* (*ETBF*), and *Porphyromonas* species (49–53), while genera such as *Roseburia*, *Ruminococcus*, *Clostridium*, *Faecalibacterium* and *Bifidobacterium* are generally depleted in CRC patients (54–56). However, these study designs cannot directly address the issue of causality, and their variable results are likely due to differing study designs, sample sizes, limited or no data on major confounders, and assay differences (57). Experimental data suggest that microbes such as *pks* (polyketide synthase)+*Escherichia coli* (*pks+Ec*), *ETBF*, *Fn*, and *SGG*, damage the gut-barrier lining and colonocyte DNA, increase proinflammatory cytokines and oxidative factors, and produce potentially carcinogenic toxins (17, 24, 58–61).

The evolving discipline of molecular pathological epidemiology (MPE) offers a powerful approach to explain the interpersonal susceptibility to carcinogenesis and to clarify the role of microbial variation and dysbiosis in the development and progression of tumour anatomical sub-sites within the colorectum and by tumour molecular sub-type features (e.g., inherited *MMR* mutations, somatic mutations such as in the *KRAS*, *APC*, *BRAF* and *PIK3CA* genes, microsatellite instability, epigenetic modifications) (62). Using taxon-specific models, a microbial GWAS study focused on the genetic contribution to microbiome variations and heritability of microbial taxa and identified genetic associations involving multiple microbial traits (63). Demonstrating the utility of such data, a recent study, using the random forest algorithm, established a novel CRC predictive model able to distinguish cancer from healthy subjects based on gut microbial single nucleotide variant markers (64). Using such integrative, multi-omic approaches that combine tumour molecular features, metagenomic, microbiome/human metabolomics, proteomics and nutrigenomics, MPE can add novel insights into the tumour pathology and host/microbiome interactions, potentially providing microbiome-modulating strategies for cancer prevention and treatment (65, 66).

GUT EPITHELIAL BARRIER FUNCTION AND COLORECTAL CANCER DEVELOPMENT

In the ‘leaky-gut’ hypothesis, there is a loss of epithelial integrity with bacterial translocation across the gut-barrier and detection by the immune system. Thereupon, the activation of cells of the innate and adaptive immune system in the lamina propria, including phagocytes and lymphocytes, are triggered to protect the gut tissue from further microbial damage (30). Commensal microbes support barrier function via colonization resistance, inhibition of certain virulence factors of pathogens, instigating immune responses and pH changes to control pathogens, and secreting antimicrobials to exclude pathogens from epithelial cells (67). The status of the mucus layer is balanced between the turnover of the goblet cells in the intestinal epithelium producing

the mucus through the action of mucin 2 glycoprotein (MUC2) (30, 68), and degradation by gut bacteria. This is likely protected by interaction between dietary fibre and gut microbiota, as recently shown for mucus and barrier integrity in a mouse model, where mucus in synthetic microbiota-colonized fibre free diet mice was five to six times thinner than colonized mice fed the fibre rich diet (68). Murine models have demonstrated that genetic ablation of *Muc2* brings bacteria into close contact with the epithelium, leading to inflammation and colon cancer (69). Strong evidence confirmed that different bacteria, including *E. coli*, *Enterococcus faecalis*, *Bacteroides vulgatus* and several *Fusobacterium* strains isolated from Crohn's disease or ulcerative colitis patients were found to invade epithelial cells *in vitro* (70–72). These findings have been confirmed *in vivo*. The ability of *Fn* to invade epithelial cells via FadA binding to CDH1 (E-cadherin) and to promote inflammation and oncogenic signalling has been demonstrated using HCT116 xenograft mice (73) and *Fn* infection in an *Apc*^{Min/+} mouse model lead to the generation of a proinflammatory microenvironment (59). It has been also observed that the Bft toxin from ETBF induces colitis and disrupts CDH1 junction, activates CTNBN1 (beta catenin) signalling, and induces IL8 secretion in murine colonic epithelial cells (CECs) (74, 75). The *E. coli* pks+ strain promoted invasive carcinoma in an azoxymethane (AOM)-treated IL-10^{-/-} mouse model, while the deletion of the pks genotoxic island from the same strain decreased tumour multiplicity and invasion in AOM/IL10^{-/-} mice (76). In addition to these bacteria, the gut symbiont *Akkermansia muciniphila* (*A. muciniphila*) has been implicated in the modulation of mucus layer thickness in the gut barrier (77, 78). Moreover, it has been shown that a recombinant protein Amuc_1434* - derived from *A. muciniphila* and expressed in an *E. coli* prokaryote cell system-inhibited the proliferation of CRC cell lines (LS174T) through the degradation of Muc2 (79). As expression of MUC2 in CRC tissue has been shown to be significantly increased compared to the normal mucosal control (80), *A. muciniphila* may inhibit development and progression of CRC through the restoration of the normal Muc2 level (79). Another study observed that the outer membrane protein Amuc_1100 increased the development of trans epithelial electrical Resistance in Caco2 cells, which indicate a role in the maintenance of gut barrier integrity (81). Animal studies confirmed that the recombinant Amuc_1100 protein improves gut barrier functionality and restrains the development of high-fat diet-induced obesity in mice (82). Furthermore, lower levels of *A. muciniphila* have been observed in faecal microbiota of colitis patients (83, 84), and biopsies of intestinal mucosa from IBD-patients compared to controls (85), further indicating the protective role of this symbiont in gut intestinal health.

It is well established that inflammation is a key environmental trigger that affects microbial composition and chronic inflammation is a hallmark of intestinal carcinogenesis (86). However, it is unclear whether inflammation is promoted by microbial dysbiosis imprinted early on by host genetics and/or diet/lifestyle, or if dysbiosis arises due to advancing inflammatory grades (58). One hypothesis for the primary role of microbial dysbiosis associated with a leaky gut in the development of several

diseases, including CRC, is that the weakness of the gut barrier causes a shift in the microbial community whereon the commensal bacteria within the epithelial cells may become pathogenic by acquiring virulence factors (29). This can disturb the epithelial structure and destabilize tight junction, causing the passage of bacterial strains. Both the bacterial invasiveness and the damage of the barrier contribute to a bacterial translocation and immune hyper activation in colonic mucosa. This condition could induce a further shift in the microbiota composition which may cause chronic inflammation and CRC onset (29) (Figure 1). Progression from damaged gut barriers through increasingly dysplastic neoplasms and finally tumorigenesis may be exacerbated in 'vulnerable' hosts, such as in obese or diabetic individuals or those with genetic predisposition to CRC (17).

MARKERS OF GUT BARRIER DAMAGE AND THEIR POTENTIAL APPLICATION IN EARLY DETECTION OF COLORECTAL CANCER

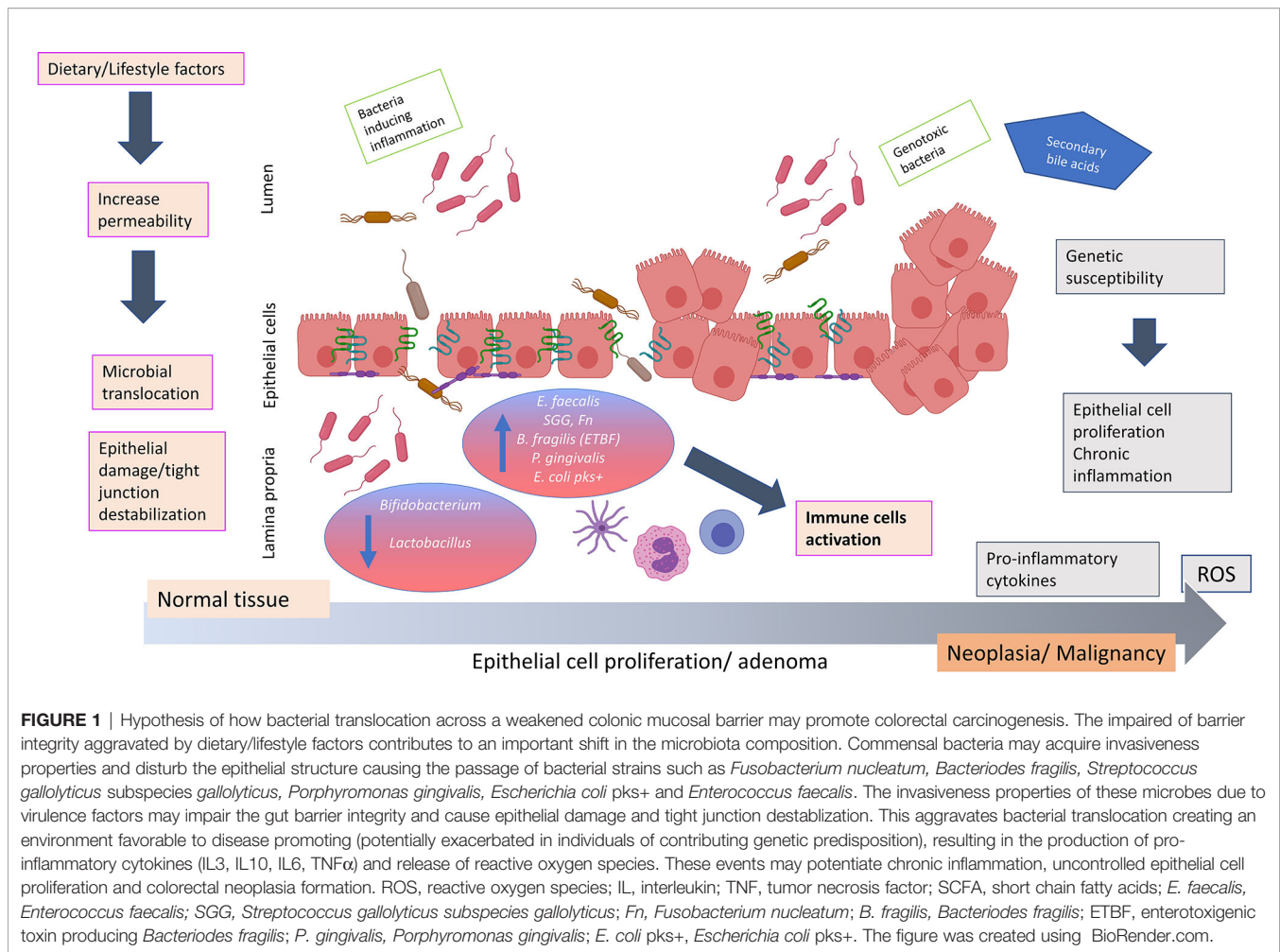
Together, the existing evidence implies a key mechanistic relationship between gut barrier integrity and microbiome composition and metabolism with colonic health (3). Indeed, levels of proteins implicated in maintaining gut barrier function have been found to be altered in inflammatory intestinal disease and CRC (87, 88). Therefore, protein markers of gut barrier function might be used as biomarkers for the early detection of CRC or stratification of the malignant potential of adenomas.

In this section we discuss promising candidate biomarkers of intestinal barrier integrity relevant to CRC. These may be classified, according to their mechanism of action at the intestinal lining, as direct or indirect biomarkers of gut-barrier functionality (Table 1).

Direct Gut Barrier Damage Markers

Proteins described within this section appear to be promising candidate biomarkers of barrier functionality as their position in the enterocytes and in the tight junction complex make them possibly more mechanistically linked to colorectal carcinogenesis mediated by gut barrier damage.

These proteins include the intestinal fatty acid binding protein (iFABP), a low molecular mass (~14-15 kDa) intracellular protein present in the epithelial cells of the intestinal mucosal layer (110). The location of iFABP facilitates its leakage into the circulation from enterocytes during intestinal damage, and results in upregulation of this protein. iFABP as a marker of enterocyte damage has been described in irritable bowel disease, necrotising colitis and coeliac disease (111). Plasma levels of iFABP were observed to be higher in patients with a severe form of ulcerative colitis compared to those with a mild form of the disease, and higher serum levels of iFABP were reported in celiac disease patients who had increased intestinal permeability compared to healthy controls (112), underlying that this protein might be used as an indicator of an extended inflammatory process (90).



Therefore, iFABP appears to be a useful candidate to evaluate gut barrier damage and inflammation in CRC (113).

Another protein that has been proposed as a direct biomarker of gut barrier integrity is zonulin [preheptoglobin-2, (92)], a protein involved in the regulation of tight junction (114). It has been reported that zonulin is upregulated during the acute phase of celiac disease (115). Increased serum and faecal levels of zonulin were also found in Crohn's patients compared to controls (91). Furthermore, an *in vivo* study observed that mice gavaged with zonulin showed increases in both small intestinal and gastroduodenal permeability compared with bovine serum albumin-treated controls (92). Asmar et al (2000) indicated that the exposure of mammalian small intestine to either pathogenic or non-pathogenic bacteria induces intraluminal zonulin secretion and increased intestinal permeability (116). Release of zonulin after bacterial exposure has been demonstrated in rat and human intestinal cell lines (IEC6 and Caco-2, respectively), and may facilitate the flushing out of microbes and toxins, participating in the innate immune response of the host to counter intestinal bacterial colonisation (117).

As it is well established that a "leaky" barrier characterized by increased tight junction permeability is a hallmark of pathological conditions including CRC, proteins involved in tight junction

structure represent an effective target for the evaluation of gut barrier status. Several tight junction proteins have been implicated in the pathogenesis of gastrointestinal cancers (118). In CRC, claudins (CLDNs) play an important role in the neoplastic transformation of premalignant epithelium tissue as demonstrated in APC^{min} mice, where the overexpression of Cldn1 induced tumour growth and progression (119). Using microarrays analysis in APC^{min} mice, the same study showed that genes regulating mucosal defence (*Muc2*, *Klf4* and *Tff3*) were downregulated while pro-inflammatory pathways, especially involving IL23/IL17 signalling, were upregulated (119). Expression of Claudin1 (CLDN1) has been shown to be increased in both high-grade dysplasia and ulcerative colitis-associated CRC tissue when compared to ulcerative colitis and normal tissue (95). Transgenic mice overexpressing intestinal Cldn1 have been used to demonstrate consequent induction of MMP9 and pERK signalling and Notch-signalling pathway activation, leading to inhibition of goblet cell differentiation, decreased *Muc2* expression and resultant mucosal inflammation (96). The junctional adhesion proteins (JAMs) are involved in the formation of the tight junction, and lower expression of JAM2 has been associated with CRC disease progression, metastasis and poor prognosis, indicating JAM2 as a tumour suppressor (97).

TABLE 1 | Candidate biomarkers for gut barrier damage in colorectal carcinogenesis.

	Candidate biomarkers	Putative function	Evidence as biomarkers	Reference
Direct measurement of intestinal damage	D-lactate and diamine oxidase (DAO)	Intestinal permeability	Plasma levels of D-lactate and DAO were found to be increased in CRC patients (n=53) compared to control (n=45)	(89)
	iFABP	Intestinal permeability	Plasma concentration levels increased in patients with severe ulcerative colitis (n = 42)	(90)
	Zonulin	Intestinal permeability	Faecal levels increased in patients with Crohn's disease (n=37) compared to control (n=40)	(91) (92)
	ZO1	Tight Junction integrity	Mice gavaged with zonulin showed increased both small intestinal and gastroduodenal permeability compared with bovine serum albumine-treated controls Lower levels in colonic mucosa of IBD patients (n=50) compared to controls (n=31) Knock out in mouse epithelial cell lines result in a loss of tight junction strands	(93) (94)
	CLDN1	Tight Junction integrity	CLDN1 expression was increased in both high-grade dysplasia and ulcerative colitis-associated CRC tissue (n=6) when compared with ulcerative colitis and normal tissue (n=39)	(95) (96)
	JAM2	Tight Junction structure	The upregulation of claudin-1 in transgenic mice induces MMP-9 and p-ERK signalling to activate Notch-signalling pathway JAM-2 expression was decreased in colorectal cell lines and CRC tissue (n=94) compared to controls (75)	(97)
	MLCK	Tight Junction regulation	Upregulated in IBD in human intestinal resection and biopsy specimens	(98)
Indirect measurement of intestinal damage	LPS and flagellin	Intestinal permeability	Serum LPS- and flagellin-specific immunoglobulin levels positively associated to CRC risk among men in a prospective cohort study. A borderline statistically significant inverse association was observed for women. Levels of LPS were found to be increased in CRC patients (n=53) compared to control (n=45)	(99) (89)
	Soluble CD14	Response to LPS exposure	Increased TLR4-CD14 expression were found in Caco-2 cell lines Plasma levels of sCD14 were positively associated with a Western diet (n=1198) in a cross-sectional study	(100) (101)
	LPS-binding protein (LBP)	Exposure to LPS	Levels of serum LBP were higher in CRC patients with cachexia (n=74) than in CRC patients without cachexia (n=78)	(102)
	Cytokine markers (e.g., IFN γ , IL10, IL12p70, IL13, IL1 β , IL2, IL4, IL6, IL8, TNF α)	Inflammation	Early barrier loss and activation of IL23/IL17-driven tumour-elicited inflammation act additively and sequentially to genetically controlled events that govern CRC development and progression in Apc ^{F^{WT}} mice IFN γ and TNF α alter barrier properties of the intestinal epithelium increasing epithelial paracellular permeability human in cell lines	(103) (104)
	Short chain fatty acids (SCFA)	Intestinal permeability	A cross sectional study observed that faecal levels of SCFA was significantly lower in CRC patients (n=19) compared to healthy control (n=16) Levels of bacteria producing SCFA assessed in stool samples were lower in CRC patient (n=15) compared to control (n=12)	(105) (106)
	Secondary bile acids	Intestinal permeability	Apc ^{min/+} mice treated with cholic acid (n=10) showed an increased intestinal permeability compared to control (n=10)	(107)
	Vitamin D and VDR	Intestinal damage	<i>In vivo</i> model of infectious colitis showed that vitamin D deficiency increased colonic hyperplasia and epithelial barrier dysfunction	(108)
	Calprotectin	Inflammation	Elevated faecal calprotectin levels associated with intestinal inflammation and IBD	(109)

CRC, colorectal cancer; iFABP, intestinal fatty acid binding protein; LPS, Lipopolysaccharide; ZO1, Zonula Occludens 1; IBD, irritable bowel syndrome; CLDN1, claudin 1; CLDN2, claudin2; JAM2, Junctional adhesive molecule 2; MLCK, myosin ligase chain kinase.

Zonula Occludens 1 (ZO1) was revealed to be essential in tight junction strand assembly as its knock out in mouse epithelial cell lines result in a loss of tight junction strands (94). Although ZO1 displacement is not sufficient to cause a barrier defect, the combination with other signalling pathways affecting tight junction structure, may contribute to a rearrangement of junctional complex, impacting on tight junction stability (114). ZO1 activity is regulated via MLCK (myosin light chain kinase) pathway and, thus, this protein represents another interesting target of gut barrier function. Expression of Mlck was reported to be increased in murine models of colitis, resulting in

dysregulation of tight junction and a severe loss of epithelial barrier function (120). Furthermore, MLCK was also shown to be up-regulated in IBD patients, implicating its involvement in altered epithelial integrity (98).

A recent study conducted by Liu et al, proposed D-lactate and diamine oxidase (DAO) as indicators to evaluate gut barrier integrity, and reported that their levels were increased in plasma from CRC patients compared to controls (89). D-lactate is produced by bacterial fermentation while DAO is an enzyme mainly produced in the small intestine and involved in the histamine metabolism (121). Both levels of D-lactate and DAO

correlated positively with levels of *Fn* and *Enterobacteriaceae* measured by qPCR in CRC stool samples, indicating that abundance of these bacterial species, implicated in CRC, may also reflect gut mucosal barrier dysfunction (89).

Indirect Gut Barrier Damage Markers

Gut microbiota play a pivotal role in homeostasis by processing nutrients from ingested food and producing numerous metabolites for the human body (107). The influence of diet on the composition of the microbiota and the exposure to metabolites produced by gut bacteria influences the intestinal epithelium and is associated with CRC risk modification (122, 123). Secondary bile acids derive from the modification of primary bile acids by gut commensals such as some *Clostridia* species in the colon (124). Several factors including high dietary fat intake have been found to increase bile acid levels (125). More recently, a study conducted in human colon carcinoma HCT116 cells showed that lithocholic acid (LCA), a secondary bile acid, stimulates IL-8 expression and induces endothelial cell proliferation and tube like formation in the tumour microenvironment, providing the strong evidence for LCA as a tumour promoter in CRC (126). *In vivo* studies in *Apc^{min/+}* mice demonstrated that deoxycholic acid (DCA) promotes the adenoma-adenocarcinoma transition and DCA-induced changes to the microbial community promoted intestinal carcinogenesis (127, 128). In 2019, the same authors demonstrated that intestinal permeability was significantly augmented in mice treated with cholic acid and that the relative abundance of *Akkermansia* and *Bacteroides* increased in the treated group compare to the control, suggesting an aggravated intestinal inflammation and impaired gut barrier function (107). In contrast with secondary bile acids, SCFA help to reinforce the colonic gut barrier through increased expression of tight junction proteins (37, 56). A cross sectional study observed that faecal levels of SCFAs were significantly lower in CRC patients compared to control subjects (105) and levels of butyrate producing bacteria, such as *Ruminococcus* spp. and *Pseudobutyrvibrio ruminis*, were found to be lower in stool samples from CRC patients in comparison to controls (106). Epidemiology studies have observed that African Americans have a higher risk of developing CRC in association with a consumption of a high fat diet (129–131), and levels of acetate, butyrate, and total SCFAs have been found to be lower in African Americans than other racial/ethnic groups, indicating a strong link between diet, SCFA levels and hence, CRC risk (132). The proposed protection from CRC development from sufficient intake of micronutrients such as selenium, vitamin D, and zinc is largely attributed to their key roles in redox and immune functioning (133–135). However, this may also be mediated by their links with modification of the microbiome and gut barrier function (136–138). For example, an *in vivo* study has demonstrated that mice supplied with a high selenium diet exhibited an abundance of *Akkermansia* and *Turicibacter* (bacteria implicated in gut barrier protection) compared to those fed with a low selenium diet (137). *In vitro* and *in vivo* studies indicate that the vitamin D signalling pathway is essential for epithelial barrier function through increasing the expression of

tight junction proteins (139). Knockout of the *VDR* gene in a Caco2 cell line showed a reduction in tight junction protein abundance and compromised function, while *Vdr^{-/-}* mice have been shown to develop severe colitis compared to *Vdr^{+/+}* mice (140). Finally, 1,25-dihydroxyvitaminD3, an active form of vitamin D, was found to inhibit enterohaemorrhagic *E. coli*-induced reduction in transepithelial electrical resistance, preserving paracellular permeability and tight junction structure in Caco2 cell lines (108). Thus, these studies indicate that vitamin D deficiency may increase susceptibility to mucosal damage.

One of the most studied indicators of gut barrier status is lipopolysaccharide (LPS), a large molecule located in the outer membrane of gram-negative bacteria. Increased permeability of the gut barrier leads to extensive translocation of microbes into the lamina propria, which is associated with higher circulating levels of LPS (141). High levels of LPS promotes metabolic endotoxemia, and may contribute to CRC development (142). A related bacterial product is represented by flagellin that acts as target of humoral immune response against infections (143). Measurement of LPS and flagellin is challenging as their presence in blood is transient. Hence, an alternative indirect method of measurement is represented by the evaluation of immunoglobulins anti-LPS and anti-flagellin, whose levels can persist longer in the human body (99). Results from the large European Prospective Investigation into Cancer and Nutrition (EPIC) showed that a combined immune response to LPS and flagellin was associated with a higher CRC risk in men, while a borderline statistically significant inverse association was observed for women (99). This contrasting result by sex might be due to immune system variation, as it is known that women have a stronger humoral/innate immune defence than men (144, 145), or, it could be attributable to a different microbiome composition (146). Levels of LPS were also measured directly in plasma; a recent study observed that levels of LPS were increased in CRC patients compared to controls, and this correlated positively with *Fn* and *Enterobacteriaceae*, indicating raised intestinal permeability in CRC cases (89). Another indicator of the amount of effective LPS present in the body is lipopolysaccharide binding protein (LBP), an acute-phase protein produced in the liver and circulating in the bloodstream (147). LBP is able to bind LPS and amplify the host immune response against it (148). A recent study showed that serum LBP levels were higher in colon cachectic carcinoma than in non-cachectic carcinoma patients, indicating this molecule as a biomarker for cancer progression and cachexia development (102).

A measurement of response to LPS is represented by the cluster of differentiation 14 (CD14) protein critical to instigating the host immune response for preserving gut barrier health. Membrane associated CD14 forms a receptor complex with TLR4 (a pattern recognition receptor) and Myeloid differentiation 2 (MD2) and plays an important role in binding LPS, as well as initiating the immune response. In healthy individuals, LPS remains in the lumen due to proper tight junction seclusion among other barrier functions. An increase in TLR4 under the presence of LPS has been associated with an increase in CD14, both of which correlated with an increase in intestinal tight junction permeability *in vitro* and *in vivo* (100). This increase in permeability further

compromises gut barrier function, potentially contributing to carcinogenesis. Furthermore, a cross sectional study observed that plasma soluble CD14 (sCD14) levels tend to increase with a Western diet, underlying the association between dietary and intestinal gut barrier dysfunction (101). Bacterial LPS also activate macrophages exposing CD14 and this evokes an inflammatory response. As a consequence, a large amount of cytokines, such as IFN γ , are produced and this can stimulate the production of other cytokines, including IL10, IL12, TNF α , in a positive feedback loop (149). A study conducted in *Apc^{fl/wt}* mice -in which tumourigenesis was caused by *Apc* allelic loss showed that early CRC- inducing genetic events may cause local loss of barrier function and entry of microbial products into the tumour microenvironment. This results in activation of IL23-producing myeloid cells, which regulate expression of downstream tumour-promoting cytokines, including IL17 and IL6 (103). Furthermore, it has been demonstrated that TNF α and IFN γ are both elevated in the mucosa of IBD patients and they induce changes in epithelial paracellular permeability, associating with tight junction protein restructuring (104). Numerous studies have showed that inflammatory reactions weaken tight junctions and further aggravate gut barrier damage (150, 151), which may over time lead to carcinogenesis.

It has been hypothesized that increased gut permeability in CRC development might be related to an increased immune response to pathobiont or pathogenic gut bacteria. This has encouraged recent serology screening studies of antibodies against microbes as markers of colorectal neoplasia and of gut barrier damage. For example, a significant association between antibody responses to SGG and CRC has been observed in several studies (152, 153). More convincingly, a study within the EPIC observed a positive association of antibody responses to SGG proteins with CRC risk in pre-diagnostic serum samples, implicating SGG serology as a potential marker for risk of developing CRC (154). Regarding the question of whether SGG infects colon tissue via a 'leaky' epithelial barrier before or after initiation of tumour development, cell line and mouse model studies of CRC demonstrated that SGG actively promotes colon cancer cell proliferation and tumour growth (52). However, as indicated in both EPIC and a recent large, prospective CRC cohort consortium in the USA (using the same analytic technique and laboratory as the contemporaneous European study (154)) concluded that, due to the long development time for colorectal tumours and the sensitivity analyses by follow-up time, SGG probably only promotes tumourigenesis after it has already begun (155). The controversy over the potential role of *H. pylori* in CRC has also been addressed in serology studies in EPIC and a larger multi-cohort study from the USA, where the antibody responses to *H. pylori* proteins, specifically HcpC and VacA, were associated in both settings with an increased risk of developing CRC (156, 157). It should be noted that in these serology studies it is impossible to know when infection first occurred, confounding an accurate sensitivity analysis of follow-up time to disease diagnosis.

All together these studies support the possible use of human proteins, such as iFABP and CD14, bacterial markers of endotoxemia and metabolites such as LPS, secondary bile acids, and SCFAs as promising biomarkers to evaluate gut barrier

damage and suggest dietary strategies and use of pre-and probiotics for CRC prevention and treatment.

FUTURE DIRECTIONS

While the role of the colonic mucosal barrier and microbial dysbiosis is increasingly recognised as pivotal in the development of colorectal carcinogenesis, there is only limited information on the use of biomarkers of colonic barrier status in association with CRC initiation and progression (as discussed in this review). Observational and experimental studies, which have thus far been more focused on other gastrointestinal diseases like IBD, are required to further assess these markers in relation to prediction of CRC development risk and progression, and to further define the mechanisms of carcinogenesis in interaction with lifestyle and microbiome factors. Although the state-of-art in CRC detection primarily remain FIT and colonoscopy, blood biomarkers of gut barrier function may have uses for minimally invasive screening applications, including as refinements to improve FIT screening accuracy. The protective role of the colonic mucosal barrier in inflammation, oxidative stress, and microbial translocation, underly the applicability of biomarkers of its dysfunction in CRC prevention and early detection.

The understanding of the interaction between host epithelium and the microbiome will provide novel information for the development of prophylactic and therapeutic interventions for CRC patients. Specifically, the inclusion of lifestyle/dietary modifications to improve gut-barrier functionality and promote gastrointestinal eubiosis could be used in both cancer prevention and patient clinical management settings. Dietary modification with increased fibre intake, alternatives to antibiotics, use of pre- and pro-biotics, and vitamin D supplementation, to restore levels of beneficial microbes such as *Lactobacillus* and *Bifidobacterium*, might rebalance gut dysbiosis and help repair barrier damage in CRC patients. Furthermore, the use of MPE, nutritional epidemiology, and microbial GWAS studies can provide novel strategies to integrate genetic variations and modifiable factors into the study of microbiome composition, gut barrier alterations, and links with tumour molecular sub-types. This multidisciplinary model, together with machine learning approaches to better mine the data, should provide new insight into the microbial-gut barrier nexus for improved personalized CRC prevention and disease treatment strategies.

CONCLUSION

The understanding of the interaction between the microbiome and the gut barrier will help to elucidate the pathogenesis of several intestinal diseases, including CRC. In this review, we have outlined candidate screening biomarkers representative of a compromised gut epithelium with a concomitant higher risk of CRC development. Although the applicability of these proteins as markers of intestinal damage relevant to CRC pathogenesis is still uncertain and the subject of current investigation, the use of biomarkers of microbial

pathogenicity and gut-barrier status may help CRC prevention, screening, and patient management. Further studies are required to assess the potential of these biomarkers for use in clinical practice.

AUTHOR CONTRIBUTIONS

Conceptualization: DH and MJ. Funding acquisition: DH and WG. Writing—original draft: FG, DH, and VR. Writing—review and editing: MJ and WG. All authors contributed to the article and approved the submitted version.

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