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Gut Microbiota in Colorectal Cancer: Associations, Mechanisms, and Clinical Approaches

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Abstract

Colorectal cancer (CRC) is associated with the presence of particular gut microbes, as observed in many metagenomic studies to date. However, in most cases, it remains difficult to disentangle their active contribution to CRC from just a bystander role. This review focuses on the mechanisms described to date by which the CRC-associated microbiota could contribute to CRC. Bacteria like *pks*$^{+}$ *Escherichia coli*, *Fusobacterium nucleatum*, or enterotoxigenic *Bacteroides fragilis* have been shown to induce mutagenesis, alter host epithelial signaling pathways, or reshape the tumor immune landscape in several experimental systems. The mechanistic roles of other bacteria, as well as newly identified fungi and viruses that are enriched in CRC, are only starting to be elucidated. Additionally, novel systems like organoids and organs-on-a-chip are emerging as powerful tools to study the direct effect of gut microbiota on healthy or tumor intestinal epithelium. Thus, the expanding knowledge of tumor-microbiota interactions holds promise for improved diagnosis and treatment of CRC.

1. GUT MICROBIOTA AND COLORECTAL CANCER

Colorectal cancer (CRC) is one of the most prevalent cancer types worldwide, with almost two million cases and one million deaths per year (Sung et al. 2021). The gut mucosa presents the highest concentration of commensal microorganisms in the human body, which intimately affect its homeostatic maintenance and are long hypothesized to be a cause of CRC. Indeed, it is estimated that 2.2 million cases of cancer are attributable to biological infectious agents, accounting for 13% of total cases (de Martel et al. 2020). However, despite all the advances in the field, no member of the gut microbiota has been officially recognized as carcinogenic by the World Health Organization to date (de Martel et al. 2020).

The advent of next-generation sequencing (NGS)-based metagenomic studies revolutionized our understanding of the human gut microbiota and its relation with CRC. 16S amplicon sequencing studies now allow for detailed characterization of the gut microbiota species in an unbiased way. Furthermore, shotgun metagenomics enable the study of functional processes encoded in the microbiome of CRC patients (Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019). This has led to the identification of a set of microbes recurrently enriched in fecal and tumor samples (**Table 1**). Bacteria such as genotoxic *pks*$^+$ *Escherichia coli*, *Fusobacterium nucleatum*, or enterotoxigenic *Bacteroides fragilis* (ETBF) are consistently associated with CRC. Additionally, genera

Table 1 Microbes commonly associated with CRC

CRC-enriched bacteria	Metagenomic studies describing microbial enrichment in CRC
Fusobacterium nucleatum	Castellarin et al. 2012; Kostic et al. 2012, 2013; Nakatsu et al. 2015; Baxter et al. 2016; Flemer et al. 2017; J. Yu et al. 2017; Thomas et al. 2019; Wirbel et al. 2019; Yachida et al. 2019; Young et al. 2021a
Parvimonas	Nakatsu et al. 2015, Baxter et al. 2016, Flemer et al. 2017, J. Yu et al. 2017, Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019, Young et al. 2021a
Peptostreptococcus	Kostic et al. 2012, Baxter et al. 2016, Flemer et al. 2017, J. Yu et al. 2017, Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019, Young et al. 2021a
Gemella	Nakatsu et al. 2015, Baxter et al. 2016, Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019, Young et al. 2021a
(*pks*$^+$) *Escherichia coli*	Arthur et al. 2012, Wu et al. 2013, Nakatsu et al. 2015, Wirbel et al. 2019, Young et al. 2021a
Porphyromonas	Baxter et al. 2016, Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019, Young et al. 2021a
Solobacterium	J. Yu et al. 2017, Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019, Young et al. 2021a
Clostridium	Flemer et al. 2017, Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019
Bilophila	Yachida et al. 2019, Nguyen et al. 2020, Young et al. 2021a
Atopobium	Yachida et al. 2019, Young et al. 2021a
Dorea	Yachida et al. 2019, Young et al. 2021a
Streptococcus	Thomas et al. 2019, Yachida et al. 2019
Prevotella	Baxter et al. 2016, Wirbel et al. 2019
Enterotoxigenic *Bacteroides fragilis*	Nakatsu et al. 2015
CRC-enriched fungi	
Basidiomycota	Gao et al. 2017, Richard et al. 2018, Coker et al. 2019
Malassezia	Gao et al. 2017, Richard et al. 2018, Coker et al. 2019
CRC-enriched viruses	
Papillomaviridae	Chen et al. 2015, Turkington et al. 2021
Polyomaviridae	Chen et al. 2015, Turkington et al. 2021
Caudovirales	Hannigan et al. 2018, Nakatsu et al. 2018

such as *Parvimonas*, *Porphyromonas*, *Peptostreptococcus*, *Gemella*, *Streptococcus*, and *Prevotella* and the Clostridiales order are also recurrently enriched in the disease (**Table 1**), although how they contribute to CRC is less studied. While most of the studies to date have focused on bacteria, the microbiota additionally comprises fungi and viruses. Indeed, opportunistic fungal pathogens such as *Malassezia* spp. (Coker et al. 2019), eukaryotic viruses such as *Papillomaviridae* or *Polyomaviridae* (Chen et al. 2015, Turkington et al. 2021), and bacteriophages such as *Caudovirales* (Hannigan et al. 2018, Nakatsu et al. 2018) are starting to be recognized as potential players in CRC development (**Table 1**).

Identifying associations of the gut microbiota with CRC and testing their ability to predict the presence of the disease (Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019) have already shown clinical potential as a diagnostic tool (Young et al. 2021a) that is valid for diverse geographical populations (Young et al. 2021b). However, while these associations have proven useful in diagnostics, they do not imply their active role in CRC development. Thus, it is important that studies using a range of biological systems, from human cell lines to mouse models and organoids, help in dissecting which microbes contribute causally to CRC, and which are mere bystanders of carcinogenesis. This will contribute to developing strategies to combat relevant CRC processes mediated by microbes (Sepich-Poore et al. 2021).

This review focuses on the current knowledge about how CRC-associated bacteria could contribute to the development of the disease, including the role of gut fungi and viruses. Additionally, we review the advances in the fields of in vitro organoid and organs-on-a-chip to study gut-microbiota interactions in the context of CRC. Finally, we discuss how this mechanistic knowledge is already shaping the clinical approach to CRC diagnostics, prognostics, and treatment.

2. MECHANISTIC INSIGHTS ON GUT BACTERIA DRIVING COLORECTAL CANCER TUMORIGENESIS

2.1. Genotoxic *pks*$^+$ *Escherichia coli*

In 2006, a landmark study identified intestinal strains of genotoxic *E. coli* harboring the *pks* operon, putting *pks*$^+$ *E. coli* on the map for CRC (Nougayrède et al. 2006). Since then, several investigations have shown the enrichment of genotoxic *pks*$^+$ *E. coli* in CRC samples (Arthur et al. 2012, Buc et al. 2013, Wirbel et al. 2019). Indeed, *pks*$^+$ strains of *E. coli* are present in approximately 60% of CRC samples compared to only 20% of healthy donors (Arthur et al. 2012, Buc et al. 2013). Additionally, *pks*$^+$ *E. coli* has been found enriched in precancerous conditions like inflammatory bowel disease (IBD) and familial adenomatous polyposis (FAP) (Arthur et al. 2012, Dejea et al. 2018).

The *pks* operon encodes a hybrid nonribosomal peptide synthetase–polyketide synthase assembly line that enables the production of the genotoxin colibactin (Faïs et al. 2018). The effects of this—highly labile—molecule on epithelial cells have been studied intensively since its discovery. They range from the induction of DNA interstrand cross-links (Bossuet-Greif et al. 2018) to cell senescence (Cougnoux et al. 2014). Additionally, *pks*$^+$ *E. coli* colonization increases DNA damage and tumor burden in mouse models of CRC (Cuevas-Ramos et al. 2010, Arthur et al. 2012, Dejea et al. 2018).

A series of publications in 2019 resolved the long-elusive structure of colibactin and enabled unprecedented insights into its DNA-damaging capabilities (Li et al. 2019, Wilson et al. 2019, Xue et al. 2019). Key insights comprise the ability of colibactin to alkylate adenosines (Wilson et al. 2019) and to form interstrand cross-links through two cyclopropane warheads (Xue et al. 2019). This causes DNA double-strand breaks (DSBs) by a copper-mediated mechanism (Li et al. 2019). In turn, this suggests a potential mechanism whereby colibactin induces cross-linking of

adenines on opposing DNA strands, leading to DSB induction and mutagenesis through improper resolution of these adducts.

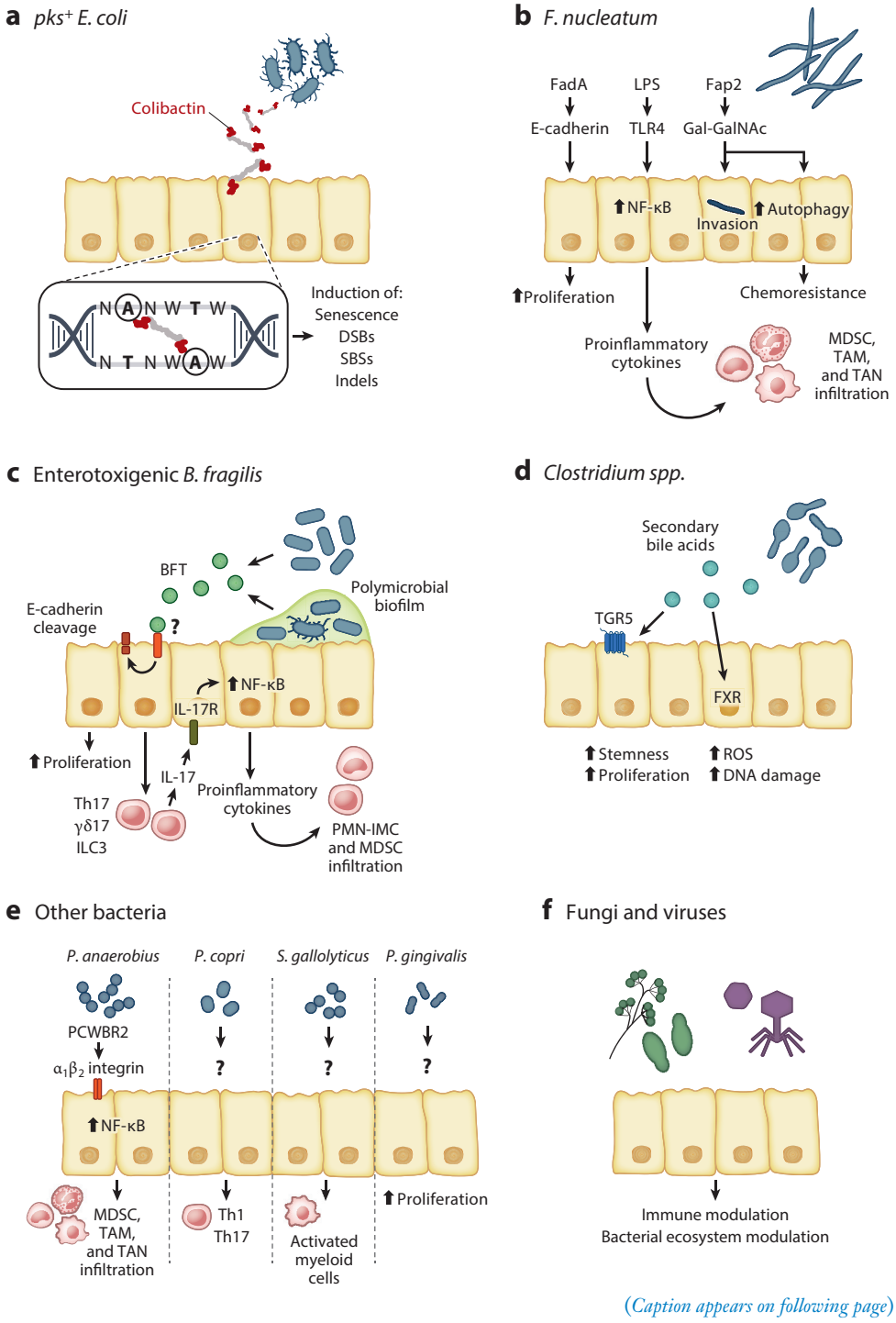
More recently, we described the ability of *pks*$^{+}$ *E. coli* to induce a genome-wide mutational signature in CRC (Pleguezuelos-Manzano et al. 2020). Mutational signatures are marks left in the genome through the effect of specific mutagens, and they can be used to explore the past exposure of tumors to these mutagens (Alexandrov et al. 2013, 2020). Thus, healthy human gut organoids, chronically exposed to the bacteria, were used to establish that—beyond DSB induction described previously—*pks*$^{+}$ *E. coli* induces genome-wide mutations in adenine-rich regions of the DNA, giving rise to readily recognizable SBS-88 and ID-18 mutational signatures. Then, this was used to identify the contribution of *pks*$^{+}$ *E. coli* to the mutational burden of CRC in patients and its potential to mutate CRC-relevant genes like *APC* (Pleguezuelos-Manzano et al. 2020). Furthermore, the genome-wide profile of DSB induced by the bacteria also showed an adenine enrichment in the damaged DNA sequence (Dziubańska-Kusibab et al. 2020). Both observations are in line with the ability of colibactin to cross-link adenines deduced in the previous structural studies (Li et al. 2019, Wilson et al. 2019, Xue et al. 2019). Another study reported Wnt-independent growth of murine intestinal organoids after a 3-h exposure to genotoxic *E. coli*, which was associated with wide-ranging genomic effects (Iftekhar et al. 2021) (**Figure 1*a***).

2.2. *Fusobacterium nucleatum*

The presence of *F. nucleatum* in CRC tumor samples was first described in two seminal studies (Castellarin et al. 2012, Kostic et al. 2012). Subsequently, metagenomic analyses from fecal (Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019) and tumor (Nakatsu et al. 2015) samples confirmed the association between *F. nucleatum* and CRC. Clinically, the presence of *F. nucleatum* correlates with left-sided, microsatellite instability–positive CRC tumors (Hamada et al. 2018, Mima et al. 2016). Interestingly, *F. nucleatum* has been found to be associated with early to late stages of CRC (Yachida et al. 2019) as well as detected in CRC-derived liver metastases (Kostic et al. 2012, Bullman et al. 2017). Furthermore, *F. nucleatum* shows no association with Lynch syndrome patients, a familial version of hypermutated CRC (Yan et al. 2020), highlighting the bacterial link with sporadic CRC.

F. nucleatum is a common member of the oral microbiota. Currently, it is thought that its translocation to colonic tumors occurs via the bloodstream (Abed et al. 2016) through the transient elevation of bacterial counts in the bloodstream after toothbrushing. Tail vein injection of *F. nucleatum* led to the specific localization of the bacteria to orthotopic colonic tumors. This tropism was mediated by the bacterial adhesin Fap2, which binds Gal-GalNAc, a sugar moiety specifically overexpressed in CRC cells (Abed et al. 2016). Other possibilities, like tumor translocation via the gastrointestinal tract, remain to be tested.

Growing evidence suggests that *F. nucleatum* can induce colon tumorigenesis. *Fusobacterium* was shown to increase proliferation in CRC cell lines (Rubinstein et al. 2013, Yang et al. 2017), colonic tumor formation in CRC mouse models (Kostic et al. 2013, Yang et al. 2017), and xenograft establishment rate (Bullman et al. 2017). It was suggested using CRC cell lines that the *F. nucleatum* surface protein FadA binds to epithelial cell-to-cell adhesion protein E-cadherin, which normally is a binding partner of the Wnt pathway protein β-catenin. As a result, this was suggested to have an impact on Wnt signaling activation levels (Rubinstein et al. 2013). Additionally, *F. nucleatum* could contribute to CRC by altering tumor sensitivity to anticancer drugs. Through a TLR4/MYD88 microRNA-mediated mechanism leading to increased autophagy, *F. nucleatum* could induce resistance to chemotherapeutics like oxaliplatin or 5-fluorouracil in cancer cell lines and CRC cell line mouse xenografts (J. Yu et al. 2017).



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Different mechanisms by which CRC-associated bacteria have been proposed to drive CRC carcinogenesis. (*a*) The production of colibactin by *pks*$^+$ *Escherichia coli* induces DNA damage (DSBs), senescence, SBSs, and short insertions/deletion mutations (indels) in CRC. (*b*) *Fusobacterium nucleatum* enhances proliferation via FadA binding to epithelial E-cadherin and the production of proinflammatory cytokines and the infiltration of MDSCs, TAMs, and TANs via LPS activation of TLR4-NF-κB. The engagement of Fap2 to epithelial Gal-GalNAc mediates bacterial invasion and increased autophagy-mediated chemoresistance. (*c*) By producing BFT, enterotoxigenic *Bacteroides fragilis* induces recruitment of Th17, γδ17, and ILC3 lymphocytes and a proinflammatory environment through the IL-17/NF-κB axis, resulting in the infiltration of proinflammatory MDSCs and PMN-IMCs. (*d*) Secondary bile acids produced by *Clostridium* spp. are proposed to induce stemness and proliferation via TGR5 and FXR activation, increased levels of ROS, and DNA damage. (*e*) Proposed mechanisms by which other bacteria could contribute to CRC: *Peptostreptococcus anaerobius* protein PCWBR2 binding to epithelial integrin induces infiltration of MDSCs, TAMs and TANs via NF-κB activation; *Prevotella copri* induces recruitment of Th1 and Th17 lymphocytes and *Streptococcus gallolyticus* recruits activated myeloid cells, although the mechanisms remain unknown; and *Porphyromonas gingivalis* increases proliferation by unknown mechanisms. (*f*) Fungi and viruses from the gut microbiota are supposed to modulate the immune and bacterial compartment. There is no conclusive evidence about whether they could directly induce CRC by their effect on epithelial cells. Abbreviations: BFT, *B. fragilis* toxin; δγ17, IL-17-producing δγ T cells; DSB, double-strand break; ILC3, type 3 innate lymphoid cells; LPS, lipopolysaccharide; MDSC, myeloid-derived suppressor cell; PMN-IMC, polymorphonuclear immature myeloid cell; ROS, reactive oxygen species; SBS, single-base substitution; TAM, tumor-associated macrophage; TAN, tumor-associated neutrophil; Th1, T helper type 1 cell; Th17, T helper type 17 cell.

Moreover, *F. nucleatum* has been proposed to induce CRC tumor inflammation. The presence of *F. nucleatum* in CRC patient samples was correlated with proinflammatory gene profiles (Kostic et al. 2013). This was confirmed later in several CRC mouse models where *F. nucleatum* induced the expression of proinflammatory cytokines and NF-κB pathway activation (Kostic et al. 2013, Yang et al. 2017). In turn, this profile led to the accumulation of tumor-associated macrophages (TAMs), dendritic cells, and myeloid-derived suppressor cells (MDSCs) (Kostic et al. 2013), which have been shown to promote tumor development and enhanced immune escape (Veglia et al. 2018). In patients, the presence of *F. nucleatum* was also inversely associated with the presence of CD3$^+$, CD3$^+$/CD4$^+$/CD45RO$^+$, and CD8$^+$ T cells (Mima et al. 2015, Serna et al. 2020, Borowsky et al. 2021). Furthermore, *F. nucleatum* has been shown to directly inhibit natural killer cell cytotoxicity by FadA-mediated binding to the TIGIT receptor (Gur et al. 2015).

F. nucleatum shows an invasive phenotype in CRC cell lines in vitro (Castellarin et al. 2012) and in CRC tumors (Bullman et al. 2017, Serna et al. 2020). However, until recently, the consequences of this invasion remained unexplored. Casasanta et al. (2020) showed that bacterial invasion into CRC cancer cell lines is dependent on Fap2 expression. Invasion was accompanied by induction of epithelial CXCL1 and IL-8 cytokine production, which in turn led to increased tumor cell migration in vitro. This, together with the presence of the bacteria in CRC liver metastases (Bullman et al. 2017, Kostic et al. 2012), suggests that by invading epithelial cells, *F. nucleatum* could mediate the metastatic process. Although this hypothesis sounds appealing, experimental evidence is not yet available (**Figure 1*b***).

2.3. Enterotoxigenic *Bacteroides fragilis*

B. fragilis is a common human gut commensal, where it contributes to a healthy intestinal tract. However, particular strains termed ETBF were identified in the 1990s for their ability to induce diarrhea and inflammation and their association with IBD (Sack et al. 1994; Prindiville et al. 2000; Basset et al. 2004; Sears et al. 2008, 2014) and, later, with FAP and CRC patients (Dejea et al. 2014, 2018; Boleij et al. 2015; Nakatsu et al. 2015). The toxigenicity of ETBF resides in the production of a matrix metalloproteinase toxin termed *B. fragilis* toxin (BFT), encoded in the genomic *bft*

locus (Sears et al. 2014). Despite its association, shotgun metagenomic analysis of BFT presence in fecal samples did not identify an association between the toxin and CRC (Wirbel et al. 2019). This observation could be confounded by differences previously observed between the detection of mucosal and fecal microbes, which was shown to particularly affect *B. fragilis* in mice (Vaga et al. 2020).

The action of ETBF through BFT has been implicated in a number of CRC-inducing mechanisms. Initially, the BFT toxin was shown to induce E-cadherin cleavage and altered Wnt pathway levels, leading to increased proliferation rates in cancer cell lines (Wu et al. 2003, 2007), similar to what has been observed for *F. nucleatum* (Rubinstein et al. 2013). Despite these observations, the specific binding partner of BFT on the epithelial cell surface has not yet been identified. Elucidating this interaction could help the development of specific inhibitors to be used as clinical drugs against ETBF and BFT.

The use of several CRC mouse models has uncovered the ability of ETBF to induce distal colon tumorigenesis via a multistep proinflammatory immune response. In $Apc^{min/+}$ and azoxymethane (AOM) mouse models, ETBF was shown to induce distal tumorigenesis one week after gut colonization (Wu et al. 2009, Chung et al. 2018, Dejea et al. 2018). Particularly, ETBF, through the action of BFT, can induce recruitment of T helper cell type 17 cells (Th17), IL-17-producing δγ T cells, and type 3 innate lymphoid cells to the colonic tumors, leading to increased IL-17 levels activating the NF-κB/STAT3 pathway in the epithelium (Wu et al. 2009, Chung et al. 2018, Dejea et al. 2018). In turn, this activates the production of proinflammatory cytokines like CXCL1, CXCL2, or CXCL5 that further recruit $CXCR2^+$ polymorphonuclear immature myeloid cells (Thiele Orberg et al. 2017) and promote their differentiation toward tumor-promoting MDSC immune cells (Chung et al. 2018).

Furthermore, ETBF is able to grow in polymicrobial bacterial biofilms, enriched in right-side CRC (Dejea et al. 2014, 2018). The significance of bacterial biofilms in CRC is not yet fully understood, but the presence of these biofilms correlates with higher levels of IL-6, pSTAT3, and proliferative cells, even in regions of normal mucosa that are in close proximity to bacterial aggregates (Dejea et al. 2014). Interestingly, Dejea et al. (2018) showed that ETBF can colocalize with *pks*$^+$ *E. coli* in polymicrobial biofilms on the polyps of FAP patients. The combined presence of ETBF and *pks*$^+$ *E. coli* increased their tumorigenicity in two mouse models of CRC. Interestingly, ETBF-induced IL-17-mediated inflammation and mucosal barrier disruption appear to facilitate the mutagenic effect of *pks*$^+$ *E. coli*. These observations suggest a cooperative contribution to CRC tumorigenesis through these combined effects (**Figure 1*c***).

2.4. Other Bacteria Associated with Colorectal Cancer Development

As the number of metagenomic studies increases, so does the evidence that there are other bacteria associated with CRC, including such genera as *Parvimonas*, *Peptostreptococcus*, *Gemella*, *Porphyromonas*, *Solobacterium*, *Clostridium*, *Bilophila*, *Atopobium*, *Dorea*, *Streptococcus*, and *Prevotella* (**Table 1**). However, proof for their active contribution to the disease is limited or absent in most cases.

Several of these bacteria have been suggested to induce inflammation of the gut. *Peptostreptococcus anaerobius* was shown to bind α1/β2 integrin of CRC cell lines via its surface protein PCWBR2 (Long et al. 2019), which induces proinflammatory cytokine production and infiltration by MDSCs, TAMs, and tumor-associated neutrophils (TANs) in $Apc^{min/+}$ mouse models. *Prevotella copri* has been shown to induce Th1 and Th17 infiltration (Yu et al. 2019). *Streptococcus gallolyticus* preferentially locates in polyps bearing mutations in APC (Aymeric et al. 2018) and induces proinflammatory cytokine secretion and infiltration of $CD11b^+$/$TLR4^+$ activated myeloid cells (Deng et al. 2020). In other AOM mouse models, the presence of *S. gallolyticus* increased tumor

burden and resulted in higher dysplasia grade (Kumar et al. 2017). Finally, *Porphyromonas gingivalis* can induce proliferation in CRC cell lines, where it displays an epithelial invasive phenotype (Mu et al. 2020). Furthermore, specific proteobacteria, including strains of *E. coli*, can produce cytolethal distending toxin, a molecule that has been shown to induce DSBs and tumorigenesis in mice (He et al. 2019).

The order Clostridiales, and particularly the ability of some *Clostridium* species to produce secondary bile acids, is strikingly associated with CRC development (Wirbel et al. 2019, Yachida et al. 2019). Members of this order are able to metabolize bile acids from the human host, producing secondary bile acids, mainly deoxycholic acid (DCA) and lithocholic acid. These are highly hydrophobic molecules that strongly activate nuclear receptors and TGR5 signaling (Jia et al. 2018). Mechanistically, secondary bile acids have been shown to induce colonic stemness and tumorigenesis through their effect on the FXR nuclear receptor in the context of a high-fat diet $Apc^{min/+}$ CRC mouse model (Fu et al. 2019). Additionally, secondary bile acids could induce chromosomal instability, potentially through increasing reactive oxygen species levels (Fu et al. 2019). Furthermore, secondary bile acids have been implicated in liver tumorigenesis. In a key study, Yoshimoto et al. (2013) demonstrated that a high-fat diet induced an enrichment of *Clostridium* in the gut microbiota and a stark increase of DCA levels in mouse. In turn, DCA promoted a senescence-associated secretory phenotype in liver stellate cells and their proinflammatory state, leading to the development of hepatocellular carcinoma (**Figure 1*d*,*e***).

3. FUNGI AND COLORECTAL CANCER DEVELOPMENT

Past metagenomic studies have focused mostly on the bacterial contribution to CRC. However, the gut fungal microbiota, or mycobiota, is also emerging as a potential player in colon tumorigenesis. In the healthy gut, there are two dominating phyla, Ascomycota (70%) and Basidiomycota (30%), with Zygomycota being detected more rarely (Hallen-Adams & Suhr 2017, Richard & Sokol 2019). To date, only a few metagenomics studies have attempted to characterize CRC fungal dysbiosis, and generally the small sample size makes it difficult to draw definitive conclusions.

CRC is characterized by an increased Basidiomycota:Ascomycota ratio, with an enrichment of *Malassezia* spp. (Gao et al. 2017, Richard et al. 2018, Coker et al. 2019) (**Table 1**). Interestingly, *Malassezia* has been demonstrated to promote pancreatic ductal adenocarcinoma after migrating from the gut lumen to the pancreas (Aykut et al. 2019), although its role in CRC needs investigation. To date, the most extensive fungi metagenomics study (Coker et al. 2019) identified a set of 14 fungal species, with potential use as CRC biomarkers, allowing healthy and early-stage CRC samples to be distinguished, as done for bacteria. The active role of fungi in CRC development remains largely unknown. Some studies have suggested that the opportunistic pathogen *Candida albicans* can alter immune cell metabolism, leading to increased inflammation and tumorigenesis (Zhu et al. 2021). Others have focused on the role of *Debaryomyces hansenii* in Crohn's disease, a subtype of IBD. *D. hansenii* is enriched in inflamed gut regions of patients and induced a type I IFN-CCL5 response that impaired wound healing in a DSS-induced colitis mouse model (Jain et al. 2021). Despite these studies, further research is required, both to validate the associations observed to date and to possibly identify new enriched fungi as the sample size and statistical power of the studies increase. This will certainly be accompanied by mechanistic studies that will deepen our understanding of the mycobiota contribution to CRC (**Figure 1*f***).

4. VIRUSES AND COLORECTAL CANCER DEVELOPMENT

While viruses have been at the forefront of infectious agents causing cancer (White et al. 2014), their role in CRC development has been harder to disentangle. While it remains challenging to

separate viral from contaminant sequencing data and to faithfully annotate viral genomes, advances in viral-like particle purification prior to sequencing and in analytical frameworks have enabled much progress on establishing the baseline human virome (Angly et al. 2005, Virgin et al. 2009, Reyes et al. 2010; Virgin 2014, Shkoporov et al. 2019).

Recent studies have reported changes in the human gut virome in CRC (Hannigan et al. 2018, Nakatsu et al. 2018, Emlet et al. 2020) and in potential precursor conditions such as IBD (Norman et al. 2015) (**Table 1**). Of note, most disease associations reported to date stem from the more abundant bacteriophages, as opposed to eukaryotic viruses infecting the gut epithelium. An increase in the overall diversity of bacteriophages (Nakatsu et al. 2018) and *Caudovirales* (Norman et al. 2015) was reported for CRC and IBD cases, respectively, but no changed diversity was observed in a further study of CRC (Hannigan et al. 2018). Identification of CRC-associated individual phage species has proven more difficult, and there has been a notably smaller effect size compared to bacteria in early studies. Nevertheless, *Inovirus*, *Tunalikevirus* (Nakatsu et al. 2018), and several other phages of the *Caudovirales*, *Siphoviridae*, and *Myoviridae* families (Hannigan et al. 2018) have been identified as most strongly associated with CRC cases.

The functional consequences of changed bacteriophage abundances on CRC are only beginning to be unraveled. The impact on bacterial communities is one of the most plausible, yet indirect, ways by which bacteriophages may impact CRC (Dahlman et al. 2021, Massimino et al. 2021). Indeed, inhibition of either pro- or antitumorigenic bacterial species is a mechanism by which bacteriophages have been shown to modulate CRC risk (Gogokhia et al. 2019, Emlet et al. 2020). A landmark study highlights that—in addition to the direct predation of specific bacterial species—a direct induction of a host immune response is a mechanism by which phages are prone to shape the CRC microbiota (Gogokhia et al. 2019). Further studies will be necessary to elucidate the functional roles of the CRC virome. The emerging insights into the roles of bacteriophages in CRC will not only reveal their contribution to cancer development but also could pave the way to bacteriophage therapies targeted at bacterial species in CRC (Turkington et al. 2021). Beyond these phage-centric studies, eukaryotic viruses such as human papillomaviruses and *Polyomaviridae* (Chen et al. 2015, Turkington et al. 2021), along with less characterized infectious agents (zur Hausen 2012, Bund et al. 2021), have been implicated in CRC development. Detection in large-scale sequencing efforts and mechanistic studies may substantiate their role in CRC development and provide new targets for CRC prevention (**Figure 1*f***).

5. ORGANOID-BASED APPROACHES AS NEW SYSTEMS TO STUDY HEALTHY COLON- AND COLORECTAL CANCER-MICROBIOTA INTERACTIONS

Developed during the past decade, adult stem cell–based organoid technology (Sato et al. 2009, 2011; Clevers, 2016) has emerged as a novel model to study CRC host-microbiota interactions (**Figure 2**). Adult stem cell–derived organoids are miniature versions of epithelial organs that can be directly established from human tissue samples. They normally grow embedded in an extracellular matrix as self-organizing 3D structures that recapitulate the cellular and molecular characteristics of the tissue from which they are derived. Since they can be derived from healthy or tumor tissue, organoids offer a great opportunity to experimentally study the microbial contribution to CRC initiation and development in a human-specific setting. Additionally, intestinal organoids can be generated from induced pluripotent stem cell intestinal organoids (Spence et al. 2011, McCauley and Wells, 2017, Múnera et al. 2017). Despite their advantages, intestinal organoids lack the presence of immune cells, which are important to shape the gut mucosa–microbe relationships and are present in more holistic approaches like mouse models.

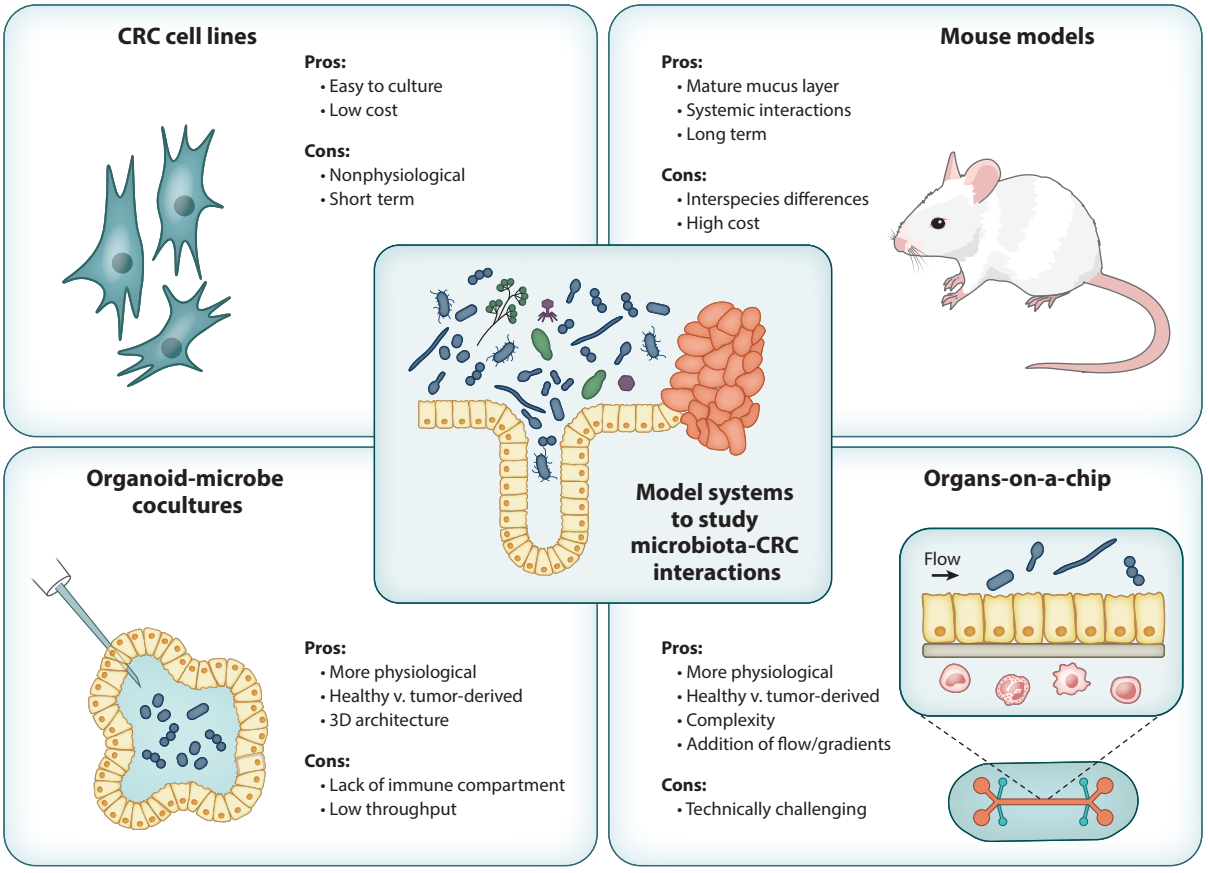


Figure 2

Different models for studying host-microbiota interactions in CRC development: CRC cell lines, mouse models, organoid-microbe cocultures, and organs-on-a-chip.

Several approaches have already been used to study the interactions between the gut and commensal microbiota using organoid cocultures, from organoid luminal microinjection and inoculation of fragmented organoids to the generation of polarized 2D organoid cultures that allow for easy apical exposure in hemi-anaerobic systems (Kim et al. 2019, Sasaki et al. 2020, Puschhof et al. 2021). More recently, organoid cocultures have been applied to CRC-associated bacteria. Mutational signatures were first linked to the genotoxic effect of bacteria, particularly that of *pks*$^+$ *E. coli*, through long-term coculture with healthy intestinal organoids (Pleguezuelos-Manzano et al. 2020). Other studies used mouse-derived intestinal organoids to further investigate the mutagenic effect of *pks*$^+$ *E. coli* (Iftekhar et al. 2021) by exposing organoid fragments in suspension to the bacteria for 3 h. Furthermore, a recent study explored the effect of an *F. nucleatum*–derived molecule cocktail on healthy colonic organoids grown as 2D monolayers (Engevik et al. 2021). This induces NF-κB activation, in line with what has been observed before in other CRC models. This suggests that if present in the colon before the onset of CRC, *F. nucleatum* could still induce inflammation and perhaps early steps of CRC tumorigenesis. Finally, murine intestinal organoids have also been used to study the effect of ETBF and BFT on healthy and tumor-derived colon organoids (Liu et al. 2020, Patterson et al. 2020).

Beside organoids, organs-on-a-chip technology holds great promise to model more complex interactions between CRC and the gut microbiota (Steinway et al. 2020) (**Figure 2**). The experimental control provided by the combination of microfluidic channels and the ability to readily install gradients of growth factors and oxygen makes organs-on-a-chip ideally suited to incorporate CRC cells, cancer-associated microbial communities, and additional microenvironmental interaction partners such as vasculature and members of the immune compartment. The past years have seen rapid progress in all of these areas (Bein et al. 2018). While intestine-on-a-chip platforms traditionally rely on the CRC cell line Caco2, new generations of chips incorporate additional cancer cell lines (Kim et al. 2012, Beaurivage et al. 2019, Carvalho et al. 2019), intestinal organoids derived from pluripotent (Workman et al. 2017, Naumovska et al. 2020) or adult (Nikolaev et al. 2020) stem cells, and even primary human biopsies (Hinman et al. 2019, Jalili-Firoozinezhad et al. 2019). Microbial communities can be cultured and repeatedly harvested from the lumen over weeks (Jalili-Firoozinezhad et al. 2019, Kim et al. 2016) and assessed for their impact on epithelial barrier integrity, the induction of cytokine release, and several other features. While the modeling of host-microbe interactions in CRC on organs-on-a-chip platforms is in its infancy, these recent advances set the stage for the rapid expansion of this field.

6. GUT MICROBIOTA IN COLORECTAL CANCER FROM A CLINICAL PERSPECTIVE

The intestinal microbiota has been historically regarded as comprising agents that could potentially cause or prevent CRC. However, it is becoming clear that the central role of microbiota in CRC has profound implications in many clinical aspects of CRC, from prevention and early diagnosis to treatment. The next sections focus on how studies of the gut microbiota are starting to affect the diagnosis, prognosis, and treatment of CRC. The gut microbiota is relevant to many other cancer types or noncancer diseases that have been reviewed previously (Sepich-Poore et al. 2021).

6.1. Intestinal Microbiota as a Colorectal Cancer Diagnostic Tool

Besides the identification of microbial associations with CRC, metagenomic studies have been shown to retrospectively predict the disease status of patients based on fecal microbial markers (Thomas et al. 2019, Wirbel et al. 2019, Yachida et al. 2019). Fecal microbial markers can discriminate not only between healthy colon and CRC, but also among early stages of the disease (Thomas et al. 2019, Yachida et al. 2019). This offers the potential to develop noninvasive, clinical diagnostic tests for patient stratification. Recently, the combination of bacterial markers with other parameters like hemoglobin presence in feces was shown to improve the predictive power of such approaches (Young et al. 2021a), which are valid across populations with different geographical and socioeconomic backgrounds (Young et al. 2021b). Additionally, recent studies suggest the potential to detect cancer onset based on microbial DNA in blood samples (Poore et al. 2020).

However, all of these approaches are based on microbial data collected during or after the onset of the disease. It will be of high interest to elucidate which bacteria are enriched prior to CRC onset. For this, large prospective cohorts can be envisioned, with longitudinal (decades-long) collection of samples from healthy individuals. During the study, some will develop the disease, which may identify microbial species that were enriched before the onset of CRC. This will allow for the implementation of stricter preventive measures and monitoring of individuals at high risk. While these kinds of cohorts already exist, they focus on healthy ageing (e.g., the Lifelines Cohort; **https://www.lifelines.nl/**) and not particularly on CRC.

Of note, the mutagenic effect of *pks*$^+$ *E. coli* has been observed in colonic crypts from healthy individuals (Lee-Six et al. 2019, Pleguezuelos-Manzano et al. 2020), indicating that these bacteria may act before the onset of the disease, most likely very early in life. This example shows that the pathogenic effect of the microbiota could occur many years, even decades, before the onset of the disease, increasing the opportunity window to intensify prevention measures for the individuals at risk. Interestingly, the same mutational patterns have been observed in head and neck and uroepithelial tumors (Boot et al. 2020, Pleguezuelos-Manzano et al. 2020), implying that the nocive effect of *pks*$^+$ bacteria might expand beyond the gut. Thus, in the case of *pks*$^+$ *E. coli*, approaches to counteract the mutagenic effect of colibactin are starting to be envisaged in the lab (Volpe et al. 2019), although their translation to the clinic remains a future goal. This exemplifies how our understanding of bacterially driven CRC mechanisms can promote the development of novel targeted therapies against their pathogenic action.

6.2. The Gut Microbiota in Colorectal Cancer Treatment

The gut microbiota can influence the outcome of cancer treatment in both positive and negative directions. During the last decade, it has become evident that immune checkpoint inhibitor treatment response (either positive or negative) is associated with particular microbial communities (Iida et al. 2013, Vétizou et al. 2015, Gopalakrishnan et al. 2018, Routy et al. 2018, Zitvogel et al. 2018). Chemotherapy is similarly influenced by the microbiota (Geller et al. 2017, T. Yu et al. 2017). *Akkermansia muciniphila* and specific members of *Bacteroidales* can promote a positive response to immune checkpoint inhibitor treatments (Vétizou et al. 2015, Routy et al. 2018). Additionally, a recent study highlighted the bacterial metabolite inosine as a key mediator enhancing immune checkpoint inhibitor responsiveness in melanoma (Mager et al. 2020). However, alterations of the microbiota have also been shown to have negative effects on treatment outcomes in diverse cancers (Gopalakrishnan et al. 2018, Routy et al. 2018). Furthermore, a recent study showed that the persistence of *F. nucleatum* in rectal tumors after postneoadjuvant chemoradiotherapy was strikingly linked to an increased risk of relapse. *F. nucleatum* presence inversely correlated with CD8$^+$ T cell infiltration, suggesting the role of the bacteria in dampening antitumor immune responses, allowing for tumor relapse (Serna et al. 2020).

There is a long-standing quest to use the gut microbiota to enhance the response to cancer treatment and to modulate its derived side effects, by using either defined probiotics, prebiotics, and postbiotics or fecal microbial transplant (FMT) (Helmink et al. 2019, McQuade et al. 2019) (**Table 2**). However, to date most of these trials aim to reduce the inflammation levels in CRC patients rather than to directly affect the treatment. Preclinical studies in mice have shown the potential of using defined bacterial communities as probiotics to boost CD8$^+$ T cell antitumor immune response (Tanoue et al. 2019). Furthermore, bacterial outer membrane vesicles have also been suggested as a potential strategy to induce antitumor immunity in CRC mouse models by inducing an IFNγ- and CXCL10-mediated immune response (Kim et al. 2017).

To date, FMT is not used as a common CRC treatment. However, an early phase I clinical trial is scheduled to assess the treatment of mismatch repair–deficient CRC patients not responding to anti-PD-1 treatment with FMT from anti-PD-1 responders (clinical trials identifier NCT04729322; **Table 2**). If positive, the results will probably serve as a first step toward the design of larger and more informative trials. Preclinical studies have shown that anti-PD-1 treatment responsiveness can be transferred via FMT from humans to mice (Routy et al. 2018). Beyond CRC, FMT has been recently employed in two trials of melanoma response to anti-PD-1 treatment (Baruch et al. 2021, Davar et al. 2021). Intriguingly, the microbial transfer from long-term immunotherapy responders induced clinical responses in 3 of 10 (Baruch et al. 2021) and 6 of

Table 2 CRC clinical trials using probiotics or fecal microbiota transplantations

NCT number[a]	Reference	*n*	Intervention	Outcome measures	Location	Status
NCT00936572	Gianotti et al. 2010	35	Probiotics: La1, BB536	Bacteria colonization and mucosal inflammation	Italy	Completed
NCT01609660	Consoli et al. 2016	33	Probiotics: *Saccharomyces boulardii*	Colonic mucosal cytokine gene expression and SCFA levels	Brazil	Completed
NCT03072641	Hibberd et al. 2017	20	Probiotics: ProBion Clinica	Changes in microbiota composition and epigenetics	Sweden	Completed
NCT03782428	Zaharuddin et al. 2019	52	Probiotics: HEXBIO	Postoperative inflammation of CRC patients	Malaysia	Completed
NCT03531606	Park et al. 2020	68	Probiotics: Mechnicov	Markers related to inflammation and levels of SCFA, Zonulin, and other cytokines	South Korea	Completed
NCT04131803	NA	140[b]	Probiotics: Bifico	Tumor size in patients receiving chemotherapy and targeted therapy combined with Bifico	China	Recruiting
NCT04021589	NA	50[b]	Probiotics: Weileshu	Progression-free survival	China	Recruiting
NCT04729322	NA	15[b]	Nonautologous FMT	Effect of FMT from dMMR CRC patients responsive to anti-PD-1 therapy to dMMR CRC patients nonresponsive to anti-PD-1 therapy	United States	Recruiting

[a]**https://clinicaltrials.gov/**.
[b]Estimated.
Abbreviations: dMMR, mismatch repair–deficient; FMT, fecal microbiota transfer; NA, not any; SCFA, short-chain fatty acids.

15 (Davar et al. 2021) patients with anti-PD-1 refractory metastatic melanoma. The insights derived from these studies, including the discovery of increased $CD68^+$ cell infiltration in the gut lamina propria (Baruch et al. 2021) and the discovery of relationships between species abundance and cytokine profiles (Davar et al. 2021), add substantial insights into the effects of FMT on immunotherapy response in cancer and are set to inspire future approaches in diverse cancer types.

7. CONCLUSIONS AND FUTURE DIRECTIONS

The development of NGS metagenomics during the last decade is initiating a revolution in our understanding of the microbiota and its association with CRC. Despite these advances, closing the gap between association and causation remains a challenging task in most cases. For some of the bacteria, particularly genotoxic *pks*$^+$ *E. coli*, *F. nucleatum*, and ETBF, there is increasing evidence of the mechanisms by which they elicit CRC tumorigenesis. These include inducing mutations, reshaping the immune landscape toward a proinflammatory protumor state, and dysregulating key epithelial signaling pathways. However, the active roles of most of the CRC-associated bacteria remain elusive. Similarly, some fungi and virus taxa have been associated with CRC, but little is known about their active contribution. Current and future research in the organoid and

organs-on-a-chip fields will generate increasingly sophisticated microbial coculture models. These will hopefully contribute toward distinguishing microbial species and communities that actively contribute to CRC development from those that play a mere bystander role and or that have mixed profiles with regard to their enrichment in and contribution to CRC. Importantly, organoids allow researchers to investigate these questions in healthy tissue and across the different stages of CRC development. Pairing these efforts with clinical studies using large patient cohorts will be necessary for these improved mechanistic insights to yield clinical relevance. These should include (*a*) prospective studies, as mentioned above, that will help identify the CRC-associated microbes that are present at precancerous stages onward, and likely play a role in tumor initiation, and (*b*) studies using cohorts designed based on new insights obtained from mechanistic in vitro and in vivo experiments. These pair whole-genome sequencing or RNA sequencing on tumor biopsies together with a microbial characterization of the samples, by either metagenomics or more targeted microbe-specific approaches, to link transcriptomic and genomic changes to bacterial effects in patients. Thus, these future research efforts will lead to a better understanding of the microbial contribution to CRC development and behavior and thereby hopefully result in refined approaches that improve CRC prevention and the diagnosis, stratification, and treatment of CRC patients.

DISCLOSURE STATEMENT

H.C. is an inventor on multiple patents held by the Dutch Royal Academy of Arts and Sciences that cover organoid technology and is a member of the board of directors of Roche. H.C.'s full disclosure is given at **https://www.uu.nl/staff/JCClevers/**. C. P.-M. and J.P. declare no conflicts of interest.

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Errata

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