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The Role of *Fusobacterium nucleatum* in Colorectal Carcinogenesis

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Abstract
Colorectal cancer (CRC) is one of the most frequent and deadly neoplasms worldwide. Genetic factors, lifestyle habits, and inflammation are important risk factors associated with CRC development. In recent years, growing evidence has supporting the significant role of the intestinal microbiome in CRC carcinogenesis. Disturbances in the healthy microbial balance, known as dysbiosis, are frequently observed in these patients. Pathogenic microorganisms that induce intestinal dysbiosis have become an important target to determine the role of bacterial infection in tumorigenesis. Interestingly, the presence of different bacterial strains, such as *Fusobacterium nucleatum*, has been detected in tissue and stool from patients with CRC and associated with substantial clinical and molecular features, as well as with patient therapy response. Therefore, understanding how the presence and levels of *F. nucleatum* strains in the gut affect the risk of CRC onset and progression may inform suitable candidates for interventions focused on modulation of this bacteria. Here we review new insights into the role of gut microbiota in CRC carcinogenesis and the clinical utility of using the detection of *F. nucleatum* in different settings such as screening, prognosis, and microbiota modulation as a means to prevent cancer, augment therapies, and reduce adverse effects of treatment.

Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second most commonly diagnosed cancer in women, with 1.8 million cases and 881,000 deaths occurring in 2018 worldwide [1]. Many studies have shown a decrease in CRC mortality rates in high-income regions such as in Northern America, Oceania, and Northern and Western Europe [2, 3]. However, an increase in both the incidence and the mortality of this tumor has been observed in less developed countries with more limited resources in Asia, Africa, and Latin America, as well as in some Eastern European countries [2, 4–7]. Several reasons have been proposed to explain this behavior in the incidence of CRC. A best treatment approach

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for the screening tests, early diagnosis, and removal of preneoplastic lesions in the USA have been implicated [2]. In rapidly transitioning countries, changes in exposure to risk factors in Latin American countries with "life style westernization," inadequate access to early detection and treatment, and delay in the diagnosis are reasons for the increase in incidence and mortality [2].

The well-established etiologic factors for CRC are: advanced age (>50 years), a personal history of CRC or adenoma, inflammatory intestinal conditions, a genetic predisposition, a diet low in fiber and high in fat and red meat, a sedentary lifestyle, diabetes, obesity, smoking, alcohol, and radiotherapy [8]. Recent and exciting studies are also reporting the importance of the gut microbiome (a collection of microorganisms, and their genes and genomes, living in association with the human gut) in this complex multifactorial disease [9–11].

The prognosis and treatment of CRC currently depend on the clinicopathological stage of the disease at the time of diagnosis. If CRC is diagnosed early (stage 0, I, or II) the 5-year survival rate is >80%, but it decreases to <10% with a late diagnosis of metastatic cancer [1]. However, the disease stage alone does not allow accurate prediction of the outcome for individual patients [12]. Therefore, a more accurate outcome prediction can contribute to personalized treatment to avoid undertreatment of patients destined to relapse or overtreatment of patients who would benefit just from surgical treatment [13].

Prognostic factors are related to the natural history of CRC that influence the recurrence rates and are frequently used to categorize patients into subgroups with different baseline relapse risks [12]. The most established clinicopathological risk factors for disease progression and death are: stage (node-positive disease), histological grade, vascular invasion, and pT4 classification. According to major guidelines, all of these variables are taken into account for selecting patients for adjuvant chemotherapy in stage II and III diseases [14]. In metastatic CRC, the extent of cancer and the aim of therapy (operable vs. inoperable metastases), and with the knowledge of CRC biology and molecular pathways, genetic and epigenetic profiles also guide the choice of systemic therapy [8].

The location of the primary tumor also seems to influence the outcome. Weiss et al. [15] showed a better outcome for left-sided diseases compared to right-sided ones in stage III but not in stage II CRC. Similarly, metastatic left-sided CRC exhibited a better outcome than right-sided CRC in previously untreated patients [16]. Apart from having a different embryological origin – proximal colon from the midgut and distal colon and rectum from the hindgut – the right colon displays peculiar differences in mucosal immunology, probably owing to differences in gut microbiota [17]. A higher concentration of eosinophils and intraepithelial T cells in the proximal colon compared with the distal colorectum has been reported [18–20]. It has been hypothesized that this could be the result of the delicate balance that immune cells have to maintain immunogenicity against pathogens and tolerance for the commensal microbiota, which is much more represented in the distal colorectum. This observation could also explain the differences in the immunological response to tumors developing in the proximal colon characterized by increased immune activity and, in turn, reflect specific differences in pathogenesis and outcomes [21–26].

CRC arises from a transition from normal mucosa to premalignant lesions, with progression to colorectal adenomas and CRC occurring over several years (Fig. 1). The most frequent path is known as the adenoma-carcinoma sequence, but CRC can also evolve through an alternative pathway in which serrated polyps, including a sessile serrated lesion and a traditional serrated adenoma replace the conventional adenoma as the precursor lesion to CRC (Fig. 1). Genetic and epigenetic alterations drive both pathways, and distinct molecular subtypes are described (Fig. 1) [8, 27–30].

Concerning the adenoma-carcinoma pathway, the initial mutations most often occur in the *adenomatous polyposis coli* (*APC*) tumor suppressor gene. Further mutations in the *KRAS* oncogene promote rapid clonal growth and an increase in cell number [8]. Additional mutations followed by clonal expansion continue, and mutations in genes such as *PIK3CA*, *SMAD4*, *TP53*, *CTNNB1*, and *BRAF* eventually result in malignancy [8, 27, 28] (Fig. 1). Not all adenomas progress to invasive cancer, although all adenomas have the capacity for malignant transformation. The pathological features of adenomas (e.g., size, type, histological grade, and the presence of dysplastic foci) are all predictive of their malignant potential; however, it is still unclear why some adenomas progress to malignancy whereas others stabilize or even regress. Noteworthy, adenomas harbor increased numbers of inflammatory cells, which are much higher than those expected in healthy colonic tissue [29].

In the serrated polyp pathway, genetic alterations involve *BRAF* and *KRAS* mutations (Fig. 1). Both *KRAS* and *BRAF* encode kinases that belong to the mitogen-activated protein kinase (MAPK) cascade that mediates cellular signaling involving cell proliferation, apoptosis, and dif-

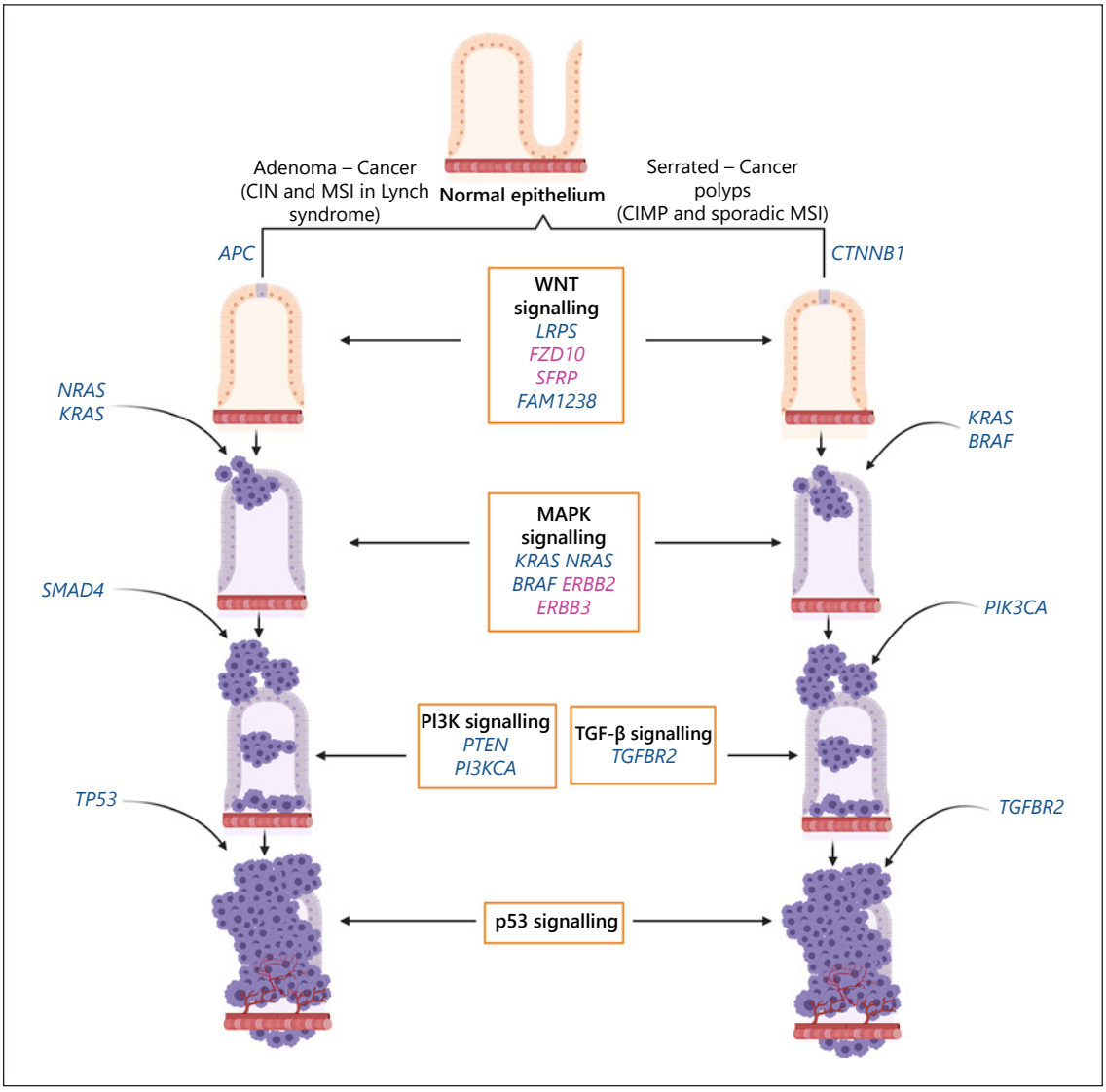

Fig. 1. Evolution of normal epithelial cells to adenocarcinoma. Two major pathways have been described from normal colon to CRC. Both involve the progression of normal colon epithelial cells to aberrant crypt foci, initiated by early and advanced polyps with later progression to early cancer and then advanced cancer. The left pathway is the traditional one, which involves the development of adenomas that progress to adenocarcinomas. The right is an alternative pathway and involves serrated polyps. The genetically (in dark blue) or epigenetically (in purple) altered genes are represented in each sequence; some genes are shared between the 2 pathways, while others are unique (*BRAF* mutations and CIMP only occur in the serrated pathway). The signalling pathways that are deregulated during the progression sequence are also shown.

ferentiation [31]. Mutations in both oncogenes are frequently found as mutually exclusive events in serrated adenocarcinoma and the precursor serrated lesions [27, 31–33]. Another molecular alteration described in serrated lesions is microsatellite instability (MSI), that is caused by the loss of mismatch repair genes, which leads to an increased susceptibility to accumulation of mutations in genes with microsatellite regions [34]. Moreover, the CpG island methylator phenotype (CIMP) is also strongly related to the serrated colorectal carcinogenesis. CpG island methylation may cause transcriptional silencing and inhibit gene expression by the binding of methyl groups to repetitive cytosine-guanine dinucleotides sequences, commonly in the promoter region [27, 31]. This epigenetic event is observed in the precursor serrated lesions and colorectal polyps [27, 31, 35] (Fig. 1).

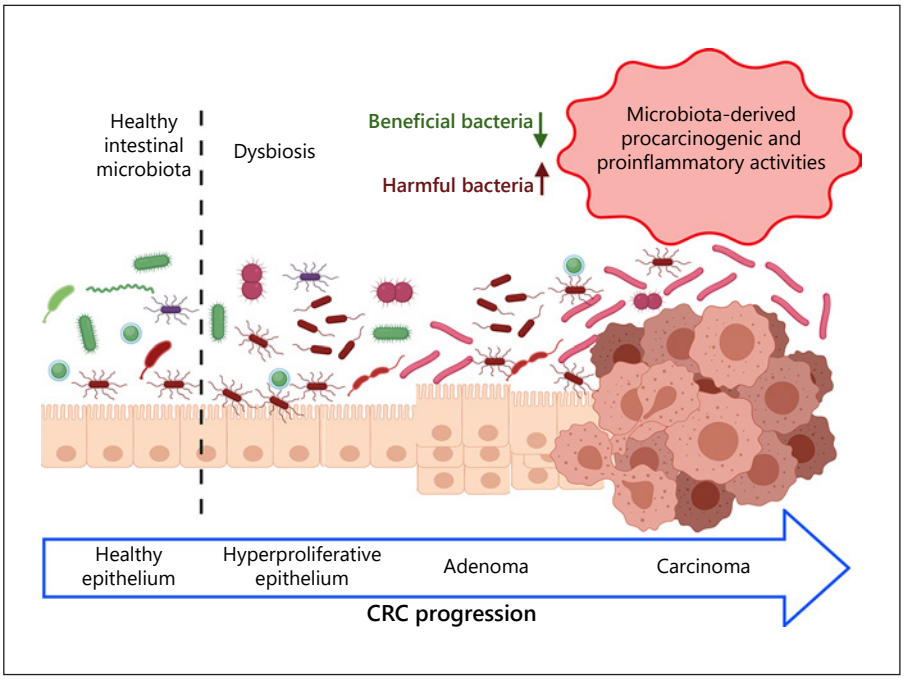

Fig. 2. CRC progression in association with gut microbial dysbiosis. In healthy epithelium of the colorectal mucosa, the majority of the endogenous bacteria in adults play a beneficial role, contributing to metabolism and nutrient absorption, and help to maintain immune structure and function (in green). Several bacterial species have been shown to exhibit proinflammatory and procarcinogenic properties, which could consequently have an impact on colorectal carcinogenesis (in red). The intestinal mucosa of CRC patients could be colonized by one or several microbes with procarcinogenic properties such as production of DNA-damaging compounds and induction of cellular proliferation, causing permeabilization of the intestinal barrier and induction of chronic inflammation, leading to the initiation of CRC.

Recently another classification was reported by the CRC Subtyping Consortium [36]. Accordingly, 4 consensus molecular subtypes (CMS) of CRC were described to resolve inconsistencies among the reported gene expression-based CRC classifications and facilitate clinical translation [36]. The classification is: CMS1, enriched for tumors with MSI, overexpression of DNA damage repair proteins, widespread hypermethylation, and high immune activation; CMS2, tumors with chromosomal instability and activation of the WNT and MYC signaling pathways; CMS3, tumors with epithelial and metabolic dysregulation; and CMS4, tumors with transforming growth factor (TGF)-β activation, stromal infiltration, and angiogenesis. Proximal CRC are enriched for CMS1 tumors, and, inversely, distal CRC are enriched for CMS2 tumors [36].

Microbiome and CRC

Recent advances in the composition and metabolism of the human microbiota have established its influence on human health [10, 37, 38]. Importantly, accumulating data suggest that the microbiota has a role in the etiology of several types of cancer by influencing inflammation, DNA damage, and apoptosis. Therefore, the involvement of the gut microbiota in CRC is an active area of research [10, 37, 38].

The majority of microorganisms are in the gut, particularly in the large intestine (colon and rectum). Different regions of the gastrointestinal tract vary widely in terms of transit time, pH, exposure to oxygen, nutrient availability, host secretions (such as bile acids and digestive enzymes), mucosal surfaces, and interactions with the immune system, with different effects on microbial colonization [39]. The large intestine contains the densest and most metabolically active microbial community ($>10^{11}$ cells per g of contents) in healthy adults, which is dominated by anaerobic bacteria that belong to 2 phyla – Firmicutes and Bacteroidetes – in addition to Actinobacteria, Proteobacteria, and Verrucomicrobia [40].

Despite substantial interindividual variation within the microbial community's composition, human studies have shown that dietary composition has an important effect on the gut microbiota [37]. Changes in fecal microbiota are detectable as early as a few days after switching between carefully controlled diets [39]. These changes can cause dysbiosis, an imbalance of the intestinal microbiome that reduces the nutrient and vitamin absorption capacity, caused by a decrease in the number of beneficial gut bacteria and an increase in disease-causing bacteria (Fig. 2) [40–42]. Compositional changes in the microbiome were recently reported in response to a switch between extreme plant-based diets (high levels of fiber and low levels of fat and protein, comprising 32 and 10% of the caloric intake, respectively) and animal-based diets

(that contained no fiber and had high levels of fat and protein, comprising 70 and 30% of the caloric intake, respectively) [43]. In response to the animal-based diet, the abundance of members of the Bacteroidetes phylum (such as *Bacteroides* spp. and *Alistipes* spp.) and *Bilophila wadsworthia* increased, whereas the number of several members of the Firmicutes phylum decreased [42].

Diet has a significant impact on the composition of the gut microbiota (besides the use of antibiotics and radiation), so dietary interventions are likely to influence the susceptibility to diseases that have a microbial component, such as CRC [42]. Although the link between fiber intake and cancer risk has been debated, recent meta-analysis studies indicate that a high-fiber intake, particularly of cereals and whole grains, is associated with a decreased risk of CRC [43]. Moreover, patients with advanced colorectal adenomas (CRC precursor lesions) are reported to have a low intake of fiber compared to healthy controls [44]. By contrast, diets rich in red and processed meat, fat, and alcohol are associated with an increased risk of CRC [45]. The lower incidence of CRC in rural native Africans compared to African Americans corresponds to a higher dietary intake of nondigestible carbohydrates relative to protein and fat, as well as major differences in the fermentation capacity of the gut microbiota [46].

Studies comparing the composition of the microbial community in patients with CRC to that of healthy subjects have been published to determine whether changes in the gut microbiota cause the disease [38]. However, as CRC develops over many years, it is a challenge to determine whether the associated changes in the gut microbiota are a consequence of diet alterations or physiology or whether they are causative. A "driver-passenger" model for CRC was proposed to distinguish between causative organisms and those that respond to disease progression [47]. The analysis of fecal samples from patients with CRC using 16S ribosomal RNA gene sequencing has shown that *Bacteroides fragilis* and several enterobacterial operational taxonomic units are enriched compared to those from healthy controls. In contrast, levels of 5 operational taxonomic units that correspond to butyrate-producing Lachnospiraceae were reduced [47]. Changes also occur in the tumor-associated microbiota, in which an increase in *Fusobacterium* spp. seems to be consistent between studies [47, 48]. Deep metatranscriptomic sequencing has more recently shown that *Leptotrichia* spp. and *Campylobacter* spp. cooccur with *Fusobacterium* spp. [49, 50]. Using a similar approach, our group recently described the richness and abundance of the microbial community in Brazilian colorectal tumor samples in comparison to adjacent normal mucosa in a small cohort of patients [49]. The results indicated a transition in the abundance of specific genera between the healthy microbiome present in normal tissue to potential oncogenic associated bacteria in CRC [49]. An increase in Fusobacteria and Proteobacteria phyla, previously associated with dysbiosis, inflammation, and CRC was observed [49, 50]. At the family level, the Enterobacteriaceae, Fusobacteriaceae, and Streptococcaceae families were distinctly enriched in CRC [49]. The CRC microbiome was also enriched by the genera *Cetobacterium*, *Odoribacter*, *Fusobacterium*, *Peptostreptococcus*, *Campylobacter*, *Aeromonas*, *Clostridium*, and *Parvimonas*. Our study corroborates the *Fusobacterium* enrichment in human CRC tissue in comparison with adjacent normal tissue, specifically, *Fusobacterium nucleatum* [49]. A recent review collected and analyzed all reliable 16S rDNA sequencing studies of intestinal microbiota from CRC patients, and found that the abundance of 32 genera belonging to the Bacteroidetes, Fusobacteria, Verrucomicrobia, Proteobacteria, Firmicutes, and Actinobacteria phyla varied significantly. Among them, 12 are genera belonging to the Bacteroidetes, Fusobacteria, Verrucomicrobia, Proteobacteria, and Firmicutes phyla, which are significantly enriched in CRC patients or tissues [51]. Among the findings, they reported a group of bacteria enriched in the CRC microenvironment such as *F. nucleatum*, *S. gallolyticus*, *C. difficile*, and *P. anaerobius*, that can directly participate in CRC development [52].

Animal models also support this link between microbiota and CRC. Clear differences in the composition of the gut microbiota have been reported in mice following tumor induction using carcinogenic agents; compared to untreated mice, *Bacteroides* spp., *Akkermansia* spp., and *Odoribacter* spp. increased, whereas *Prevotella* spp. and *Porphyromonas* spp. decreased [53]. The growing list of potentially carcinogenic bacteria provides support for the hypothesis that tumorigenesis is driven by mechanisms and/or pathways that are common to many bacterial groups rather than a single organism [51].

A decrease in microbial diversity, including a reduction of specific bacterial genera like *Clostridium* and *Bacteroides*, has been observed in CRC tissues [48]. This major shift in the microbial community structure may be due to the inhospitable tumor environment in which the rapidly growing tumor cells are competing for nutrients and the infiltrating immune cells are producing inflammatory compounds, like reactive nitrogen stress and reactive oxygen stress, that can be toxic to microbes. On the

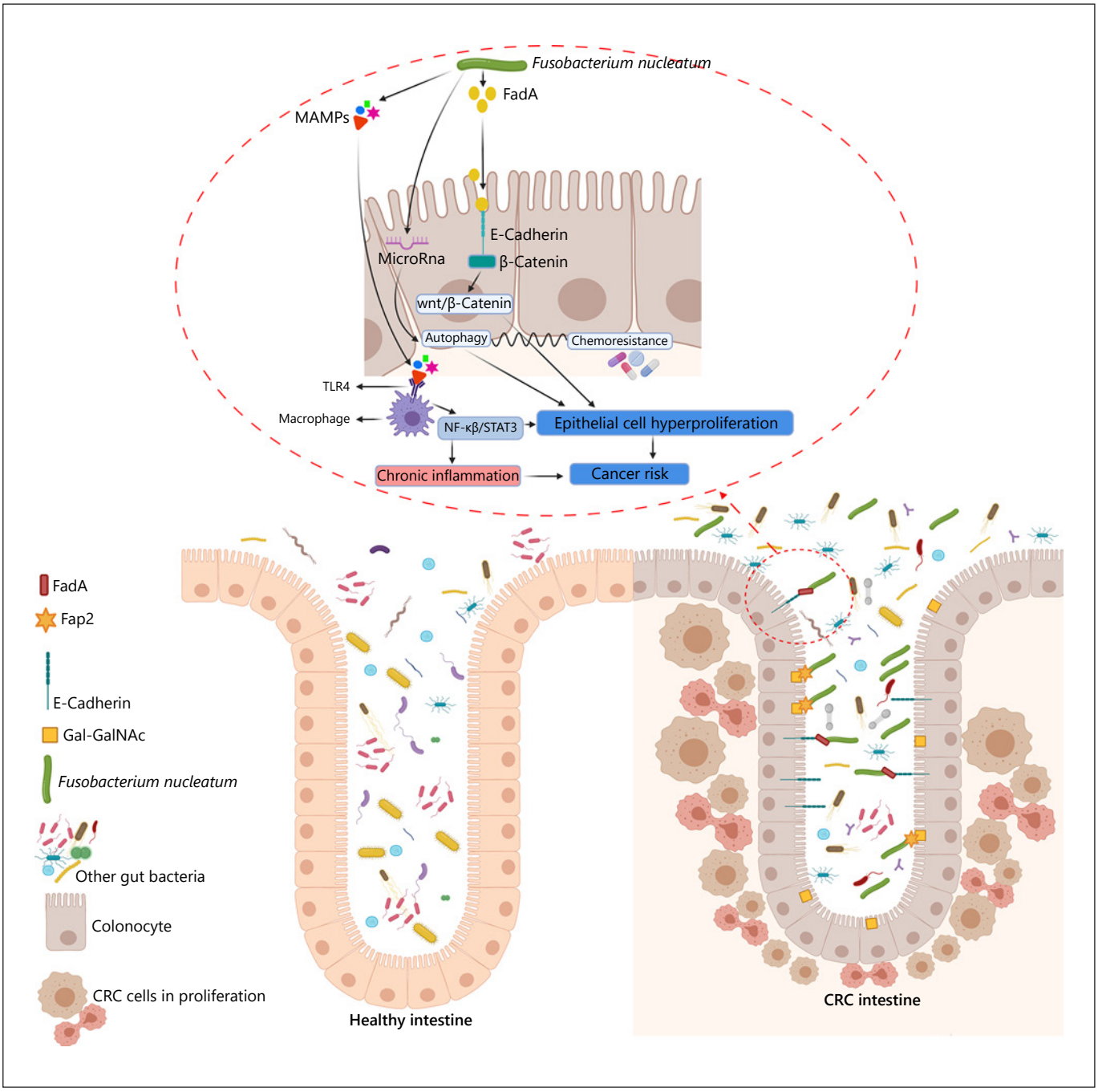

Fig. 3. *F. nucleatum*-associated mechanisms involved in the development and progression of CRC. *F. nucleatum* can directly bind through its surface protein Fap2 to Gal-GalNAc that is overexpressed in CRC cells, and *F. nucleatum* adhesin-A (FadA) can help *Fn* adhere to E-cadherin of intestinal epithelial cells, resulting in specific *F. nucleatum* accumulation in CRC tissues; binding to E-cadherin activates β-catenin, and the Wnt/β-catenin pathway, leading to uncontrolled cell growth. *F. nucleatum* also contributes to proinflammatory effects via the recognition of microbe-associated molecular patterns (MAMP) by TLR, leading to activation of the NF-κB or STAT3 pathway and accelerating CRC development and progression. *F. nucleatum* can also modulate autophagy of intestinal epithelial cells by activation of regulatory microRNA, which is interconnected with a chemoresistance mechanism.

other hand, enrichment in the highly heterogeneous *Fusobacterium* genera has also been observed in various independent studies in CRC-involved tissue relative to adjacent nonneoplastic tissue [48, 54, 55]. The dysbiosis, the decrease in beneficial gut bacteria, and the increase in harmful bacteria, which exhibit proinflammatory and procarcinogenic properties during the colorectal carcinogenesis process, is illustrated in Figure 2.

F. nucleatum and Colorectal Carcinogenesis

F. nucleatum is a gram-negative bacteria and a normal constituent of the human oral cavity [56]. As a resident member of the oral microbiota, *F. nucleatum* has been primarily studied for its role in periodontal health and disease [56] and its many adhesins that mediate binding to abiotic surfaces, host cells, or other microorganisms [57–61]. This species has been recognized as an opportunistic pathogen involved not only in inflammation process such as periodontitis, inflammatory bowel disease, pancreatic abscess, premature, and hepatic abscess but also in cancer development and progression including CRC and oral cancer [51].

While *Fusobacterium* spp. are rarely detected in the gut of healthy individuals, they can be isolated from patients with inflammatory bowel disease [61], further supporting a link between fusobacteria and an inflamed colonic environment. Some studies in human CRC samples have revealed that *Fusobacterium* ssp. often cooccur with other gram-negative anaerobes, including *Campylobacter* ssp., creating what is called a biofilm [57]. Biofilm is a microbial-derived sessile community characterized by cells irreversibly attached to a substratum or interface to each other, embedded in a matrix of extracellular polymeric substances that they have produced [61]. *F. nucleatum* seems to have an essential role in oral cavity biofilms and may be a pioneer microbe that creates a physical and metabolic structure that supports wide microbial shifts in evolving tumors over time [57]. Recent work examining microbial community changes across CRC progression has sought to identify strong microbial networks that might function together at different stages during tumor formation [61].

The role of *F. nucleatum* in colonic carcinogenesis has been implicated in progression of advanced colorectal carcinoma. Previous studies have suggested that this species promotes colon carcinogenesis through inhibition of proliferation and induction of apoptosis in T cells [62]. Other studies revealed that elevated levels of *F. nucleatum* in colon tissue are inversely correlated with the density of T CD3+ cells and strongly associated with MSI and a CpG methylator phenotype [49, 50, 63].

One recurrent question is: how can *Fusobacterium* colonize colorectal tissues, being an oral bacteria? Some studies have suggested a mechanism involving an *F. nucleatum* adhesion protein called Fap2 that binds to D-galactose-β(1–3)-N-acetyl-D-galactosamine (Gal-GalNAc), which is over-represented in CRC cells [62]. Further, *Fusobacterium* adhesin A (FadA) can help *F. nucleatum* adhere to E-cadherin of intestinal epithelial cells [64]. *F. nucleatum* can thus selectively colonize CRC tissues through Fap2 and FadA and, when in contact with intestinal epithelial cells, activate β-catenin, leading to uncontrolled cell growth and acquisition of a stem cell-like phenotype (Fig. 3) [51, 64, 65]. Experimentally, when injected into the tail veins of precancerous and malignant CRC mouse models, *F. nucleatum* was also found to colonize colorectal tissue, suggesting that *F. nucleatum* uses a hematogeneous route to reach colorectal tissue from the oral cavity [65]. In ApcMin/+ mouse models, *F. nucleatum* increased tumor multiplicity, activated the nuclear factor-κ-B (NF-κB) pathway, and drove myeloid cell infiltration into tumors, generating a proinflammatory environment that promoted CRC development [62]. Moreover, *F. nucleatum* could increase the proliferation of CRC cells and tumor development via TLR4 signaling activation [66].

Several studies show *F. nucleatum* enrichment in colorectal tissues with high-grade dysplasia and adenomas [55, 67]. The virulence factor FadA can promote E-cadherin-mediated tumor growth and induce the host to produce proinflammatory cytokines, with *fadA* gene levels in colon tissues from patients with adenomas and adenocarcinomas being >10–100 folds higher than those in healthy individuals [64]. The Fap2 protein of *F. nucleatum* can also directly interact with the T-cell immunoreceptor with the Ig and ITIM domains (TIGIT) protein, inhibiting natural killer cell cytotoxicity [68].

Potential Use of *F. nucleatum* in CRC Management

Studies demonstrating the presence and levels of *F. nucleatum* DNA in tumor tissue and even in stool samples have raised its application in a clinical setting, as a valuable biomarker for CRC diagnosis, prognosis, and treatment management. Next, we describe and exemplify some of these possible applications; they are summarized in Table 1.

Table 1. Summary of studies that reported the association between *F. nucleatum* and CRC risk, prognostication, or therapy response

Study	Population	Methodology	Sample size	Specimen type	Findings
Risk factor					
Castellarin et al. [52]	Canada	qPCR	99 CRC and ANT	FFT	*Fn* 415× ↑ in CRC than in ANT
Kostic et al. [62]	USA and UK	qPCR	27 CRC, 28 AD, and 31 HC	FFT and ST	*Fn* ↑ in FFT AD than in HC *Fn* ↑ in FFT CCR than in AD *Fn* ↑ in ST of AD than in HC
Flanagan et al. [67]	Europe (IE, CZ, DE)	qPCR	122 CRC, 52 AD, and 25 HC	FFT and ST	*Fn* ↑ in FFT AD than in HC *Fn* ↑ in ST of CRC than in AD *Fn* ↑ in ST of AD than in HC
Ito et al. [70]	Japan	qPCR	511 CRC, 343 serrated lesions, and 122 nonserrated adenomas	FFPE	*Fn* positivity ↑ in CIMP-H lesions *Fn* positivity ↑ according to a ↑ histological grade
Fukugaiti et al. [75]	Brazil	qPCR	7 CRC and 10 HC	ST	*Fn* ↑ in ST of CRC than in HC
Li et al. [74]	China	qPCR and FISH	101 CRC and 101 NAT	FFT and FFPE	*Fn* ↑ in CRC than in NAT
Wong et al. [71]	China	qPCR	104 CRC, 103 AD, and 102 HC	ST	*Fn* ↑ in ST CRC than in AD *Fn* ↑ in ST CRA than in HC *Fn* + FIT ↑ sensitivity than FIT for AD and CRC detection
Suehiro et al. [58]	Japan	ddPCR	60 HC, 30 AD/CIS, and 158 CRC	ST	*Fn* ↑ in nonadvanced AD than in HC *Fn* ↑ in advanced AD/CIS than in HC *Fn* ↑ in the CRC group than in HC
Guo et al. [76]	China	qPCR	367 CRC, 100 NGC, and 258 HC	ST	*Fn* ↑ sensitivity and specificity to CRC *Fn* / probiotic bacteria, ↑ CRC diagnostic
Proença et al. [72]	Brazil	qPCR	27 AD/NAT and 43 CRC/NAT	FFT	*Fn* ↑ in FFT AD than in NAT *Fn* ↑ in FFT CRC than in NAT *Fn* ↑ in FFT CRC than in AD
Tunsjø et al. [73]	Norway	qPCR	25 CRC, 25 AD, and 22 HC	ST and FFT	*Fn* ↑ in ST CRC than in AD and HC *Fn* ↑ in FFT CRC than in AD and HC
de Carvalho et al. [49]	Brazil	NGS and qPCR	152 CRC and 57 NAT	FFT and FFPE	*Fn* ↑ in CRC than in NAT
Butt et al. [81]	Europe	Flow cytometery	485 CRC and 485 HC	SR	No *Fn* protein antibody associations to CRC
Liu et al. [78]	China	qPCR	53 CRC and 45 HC	ST and Blood	*Fn* ↑ CRC than in HC
Grobbee et al. [77]	The Netherlands	qPCR	200 FIT +	ST	*Fn* ↑ CRC and high-grade dysplasia
Prognostic factor					
Flanagan et al. [67]	Europe (IE, CZ, DE)	qPCR	122 CRC, 52 AD, 31 ST, and 25 HC	FFT and ST	CRC patients with ↓ *Fn* levels had a longer OS than patients with ↑ *Fn* levels
Mima et al. [84]	USA	qPCR	1,069 CRC	FFPE	*Fn* ↑ with a ↑ cancer-specific mortality *Fn* ↑ in MSI-H, CIMP, and *BRAF* mutation
Li et al. [74]	China	qPCR and FISH	101 CRC and 101 NAT	FFT and FFPE	*Fn* ↑ with lymph node metastasis
Sun et al. [88]	China	qPCR	152 CRC/NAT	FFT	*Fn* ↑ with CRC tissue, poorer tumor differentiation, deeper tumor invasion, lymph node metastasis, distant metastasis, and an advanced TNM stage
Yu et al. [91]	China	qPCR	296 CRC	FFT and FFPE	*Fn* ↑ in recurrent vs. nonrecurrent patients
Suehiro et al. [58]	Japan	ddPCR	60 HC, 30 AD/CIS, and 158 CRC	ST	*Fn* ↑ in CRC stages I–IV than in stage I
Yan et al. [87]	Japan	qRT-PCR	280 stage III/IV CRC	FFPE	*Fn* ↑ with tumor invasion, lymph node metastasis status, and distant metastasis
Lee et al. [83]	Korea	qPCR	242 CRC/NAT	FFT and FFPE	*Fn* ↑ in MSI-H and CIMP-H *Fn* ↑ with a ↓ survival in metastatic CRC
Yamaoka et al. [89]	Japan	ddPCR	100 CRC and 72 NAT	FFT	*Fn* ↑ in stage IV CRC than in NAT

Table 1 (continued)

Study	Population	Methodology	Sample size	Specimen type	Findings
Chen et al. [86]	China	qPCR	91 CRC	FFPE	*Fn* ↑ lymph node metastasis, neurological invasion, vascular tumor, and MSI *Fn* ↑ associated with a shorter survival time
Kunzmann et al. [85]	Czech Republic	qPCR	190 CRC/NAT	FFT	*Fn* ↑ associated with a poorer prognosis and OS
de Carvalho et al. [49]	Brazil	NGS and qPCR	152 CRC/57 NAT	FFT and FFPE	*Fn* ↑ in proximal, depth of invasion, ↑ clinical stages, poor differentiation, MSI-H and *BRAF* mutation *Fn* ↑ correlated with a worse patient OS
Serna et al. [99]	Spain	RNA-ISH and qPCR	143 RCA	FFPE	*Fn* ↑ risk of relapse in nCRT
Therapy response					
Yu et al. [91]	China	qPCR	296 CRC, cell lines and mice	FFT and FFPE	*Fn* contributed to chemoresistance to oxaliplatin and 5-fluorouracil
Yan et al. [87]	Japan	qRT-PCR	280 stage III/IV CRC	FFPE	*Fn* ↓ patients benefit from chemotherapy (FOLFOX scheme)
Zhang et al. [92]	China	qPCR	94 CRC, cell lines and mice	FFPE	*Fn* ↑ *BIRC3* via the TLR4/NF-κB pathway *Fn* ↑ resistance to 5-fluorouracil by ↑of *BIRC3* preclinically *Fn* ↑ resistance to 5-fluorouracil CRC patients

Fn, *F. nucleatum*; AD, adenomas; CIS, carcinoma in situ; HC, healthy controls; ANT, adjacent normal tissue; FFT, fresh frozen tissue; NGC, nongastrointestinal cancer; FFPE, formalin-fixed and paraffin-embedded; FIT, fecal immunochemical test; ST, stool (fecal); qPCR, quantitative real time polymerase chain reaction; FISH, fluorescence in situ hybridization; ddPCR, droplet digital polymerase chain reaction, qRT-PCR, quantitative reverse transcription polymerase chain reaction; NGS, next-generation sequencing (16S rRNA sequencing); SR, serum; RNA-ISH, RNA in situ hybridization; RCA, rectal cancer; rDNA, ribosomal DNA; AUC, area under the curve; OS, overall survival; MMR, mismatch repair; nCRT, postneoadjuvant chemoradiotherapy.

FN as a Risk Factor and Marker to Improve Diagnostic Efficiency

The identification of biomarkers that can accurately identify individuals at risk for development of precursor lesions or cancer is extremely important to increase the chances of early detection and treatment success, thus improving survival rates [69]. Besides, the identification of noninvasive cancer biomarkers is highly needed to decrease costs and discomfort for patients [9]. In this scenario, fecal samples, which can provide information of the mucosal microbial environment without the need for invasive procedures such as colonoscopies, seem to be an exciting approach [67, 68]. The identification of *F. nucleatum* enrichment during the premalignant stage of colorectal carcinogenesis begins to build a case for *F. nucleatum* as a pathogenic gut bacteria and a risk factor for CRC.

Six studies evaluating premalignant lesions from a total of 624 patients have suggested *Fn* as a promising biomarker for CRC development risk, since high levels of *Fn* have been consistently reported in patients with adenomas compared to healthy subjects [58, 62, 67, 70–73]. Furthermore, 9 studies including a total of 1,238 CRC also showed high levels of *Fn* in CRC tissue compared to normal adjacent tissue from the same patients or compared to tissue from healthy subjects [49, 52, 58, 62, 67, 70, 72–74] (Table 1).The seminal study of Ito et al. [70] investigated the presence of *Fn* in premalignant colorectal lesions and CRC and found increasing levels of *Fn* from premalignant to cancer lesions and from the sigmoid colon to the cecum and in higher histological grades. Moreover, *Fn* was more frequently detected in CIMP-high premalignant lesions than in CIMP-low/zero lesions, suggesting that it may contribute to the progression of colorectal neoplasia [70]. This trend was consistently observed in different populations from different parts of the world and using distinct methodologies (Table 1).

Importantly, similar observations have been reported not only in preneoplastic and neoplastic tissue but also in stool samples, suggesting *Fn* detection as a potential noninvasive marker for CRC screening. Stool analyses from 9 studies with 863 CRC patients, 238 premalignant le-

sions patients, and 608 controls (no CRC patients or healthy subjects) revealed significant high *Fn* levels in stool from patients with CRC and premalignant lesions compared to controls [58, 62, 67, 71, 73, 75–78] (Table 1).

Particularly, in the subset of the 8 previous mentioned studies, the authors investigated *Fn* levels in DNA isolated directly from stool samples and demonstrated that *Fn* was significantly present at higher levels in samples from subjects with premalignant lesions than in samples from healthy subjects [58, 62, 71, 73, 76, 78] and in stool from CRC patients compared to stool from those with adenomas or controls [67, 75]. Liu et al. [78], besides finding higher levels of *Fn* DNA in stool from CRC patients than healthy subjects, also observed increased plasma levels of biomarkers of gut mucosal barrier dysfunction, suggesting their potential to monitor the development and progression of CRC [78] (Table 1).

A fecal immunochemical test is recommended as a noninvasive screening test; however, it shows a low sensitivity for advanced adenoma [30]. Wong et al. [71], besides showing a significant higher abundance of *Fn* in fecal samples from CRC or advanced adenoma patients compared to the control group, also showed that combining *Fn* positivity with fecal immunochemical test results significantly increased the sensitivity for advanced adenomas and CRC detection. Also, in the same line, Guo et al. [76] showed that *Fn* could play a role in microbiota dysbiosis by secreting antagonistic substances against probiotics bacteria and found that the altered ratio of *Fn* to important probiotic bacteria was identified as a valuable biomarker for screening, increasing the sensitivity and specificity for CRC early detection. Moreover, Grobbee et al. [77] found higher levels of *Fn* in CRC patients and those with high-grade dysplasia lesions compared to those who had normal mucosa under colonoscopy examination (Table 1).

Altogether, these results indicate that stool-based quantification of *Fn* might have an impact on the diagnostic, serving as a novel noninvasive diagnostic biomarker for CRC [69]. However, the feasibility of using the detection of *Fusobacteria* in fecal samples for early detection of colorectal lesions requires further validation. Similarly, other screening approaches, such as detection of antibodies that may arise in response to *F. nucleatum*-specific antigens, may allow paths for more studies to benefit patients with CRC [10, 79, 80]. Accordingly, Butt et al. [81] showed no *Fn* protein antibodies in the blood of CRC patients, suggesting that future prospective studies, specifically detecting *F. nucleatum* in stool or tissue biopsies, are needed to complement their findings (Table 1).

FN as a Prognostic Factor

Besides finding significant changes between normal and cancer tissue, as well as premalignant lesions, an array of studies consistently showed the potential prognostic biomarker utility of *F. nucleatum* (Table 1).

The association between *Fn* levels and a more aggressive disease was recently confirmed by a meta-analysis that depicted previous findings of a poorer survival rate in CRC patients with a high versus a low *F. nucleatum* abundance [82]. Lee et al. [83] found high *Fn* levels to be an independent negative prognostic factor for OS in metastatic CRC. Similarly, Flanagan et al. [67] found that patients with low *F. nucleatum* levels had a significantly longer overall survival than patients with moderate and high *Fn* levels, highlighting the potential of Fn detection as a prognostic determinant in CRC patients (Table 1). Mima et al. [84] assayed a large cohort of CRC patients (n = 1,069) for the presence of *F. nucleatum* DNA in cancer tissue and observed that the amount of *F. nucleatum* was associated with a shorter survival, suggesting its role as a potential prognostic biomarker. Although they also found an association between high levels of *F. nucleatum* in colorectal tumor tissues with poorer overall survival, Kunzmann et al. [85] observed that the inclusion of *F. nucleatum* in risk prediction models did not improve the ability to identify patients who died, with disease pathology staging playing a more important role.

Besides investigating the association of *Fn* levels with patient survival, similar studies also measured the impact in other molecular and clinicopathological variables (Table 1). *Fn* was consistently associated with MSI-H [51, 84, 86], CIMP [83, 84], and *BRAF* mutations [51, 84]. Additionally, higher *Fn* levels were also associated with patients with positive lymph nodes metastases [74, 86–88], neurological invasion [86] and vascular tumor thrombus [86], a higher stage disease, and distant metastasis [58, 87–89] (Table 1).

These studies comprehensively show the impact of high levels of *F. nucleatum* on the prognosis of CRC, suggesting its use as a biomarker to anticipate patient outcomes (Table 1).

FN and Therapy Response Prediction and Perspectives for Treatment Approaches

Importantly, some studies are addressing the impact of *Fn* in CRC patient response to adjuvant therapies [90] (Table 1).

Evidence suggest a potential relationship between *Fn* and resistance to 5-fluorouracil chemotherapy (which is a standard treatment for advanced CRC patients) and ox-

aliplatin and no response to immunotherapy [91–94]. Zhang et al. [92] also observed, in advanced CRC patients who received standard 5- fluorouracil-based adjuvant chemotherapy, that a high *Fn* abundance is an independent risk factor for recurrence. Yan et al. [87] observed that patients with low levels of *Fn* benefit from adjuvant chemotherapy more than those with high *Fn* levels, in terms of disease-free survival, and it may be an effective adjuvant approach for preventing CRC metastasis and chemotherapy resistance (Table 1).

Other studies also investigated pathways that are activated by *Fn* and could be targets for treatment of *Fn*-related CRC [94, 95]. The development of anti-Fap2 antibodies could be used to treat *Fn*-positive tumors and may allow restoration of antitumoral immune detection and response [10, 68]. Moreover, *Fn*-enriched tumors demonstrate increased myeloid cells; this treatment would block myeloid cell migration and differentiation and can drive myeloid infiltration and intratumoral function, reducing inflammation and the development of CRC [10].

In addition to these strategies, modification of the tumor microenvironment by methods which stimulate the patient's own immune system to fight tumor cells by fecal transplantation is a novel and important topic that will shape CRC management in the future [10, 96–98].

Conclusion

It is clear that the bacteria on the surface of the gastrointestinal mucosa dramatically influence the development and progression of CRC. However, its contribution to carcinogenesis remains complex. Herein, we gave special attention to *F. nucleatum* and addressed how it can modulate CRC development and alter the immune response against cancer. The directions for future research will aim to understand the diverse contributions of bacteria and their metabolites to carcinogenesis. Modulation of the microbiota has already been proven to be of high clinical relevance and it will continue to open new paths for the prevention and treatment of CRC.

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Statement of Ethics

This work is exempt from the requirement of ethical committee approval because it is a review article.

Conflict of Interest Statement

The authors have no conflict of interests to declare.

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Author Contributions

R.M.R. conceived the original idea and led the writing. J.G.D. wrote this article and made the figures. A.C.C., D.P.G., and R.M.R. contributed with ideas, editing, and review of this paper. All of the authors critically reviewed and approved the final version of this work.

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