LITERATURE REVIEW

On Patients with Colorectal Cancer:

Associations, prognosis, survival and effects of therapy performed in patients with Colorectal Cancer and the intestinal microbiota

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Keywords: Colorectal cancer; familial adenomatous polyposis; Fusobacterium nucleatum; intestinal microbiota.

ABSTRACT

Background: Colorectal cancer (CRC) is one of the most common cancers in the Western world, with approximately 1.2 million people diagnosed worldwide each year. Most CRCs are sporadic, resulting from chromosome instability and dysplasia of adenomas to carcinomas. At the same time, the hereditary syndromes of familial adenomatous polyposis (FAP) and hereditary nonpolyposis colpos (HNPCC) arise due to germline mutations in the APC gene and the microsatellite instability pathway. Dysbiosis and associated chronic inflammation have previously been implicated in inflammatory bowel disease, irritable bowel syndrome, and type 2 diabetes mellitus. They are now known to facilitate carcinogenesis in CRC through genetic and epigenetic mechanisms. The dysbiotic bacterium primarily implicated in CRC is Fusobacterium nucleatum, associated with microsatellite instability and lymph node metastasis in clinical trials. Recent clinical studies have also suggested that they may affect prognosis, which, if established, could potentially signal a new frontier in the diagnosis, evaluation and therapeutic management of CRC..

Objectives: To systematically review the literature to gather evidence investigating the associations between gut microbiota and CRC, colorectal adenomas, CRC tumour site, CRC stage, prognosis and survival, and the effect of current therapy performed for the treatment of CRC.

Methodology: A systematic review of the published literature.

Results: 53 studies were considered relevant for inclusion, covering a total of 5167 CRC patients, of which 3754 were tested through mucosal tissue samples, 1072 through stool samples and 341 through a combination.

Conclusion: There is a significant association between gut microbiome and CRC, with emphasis on Fusobacterium (genus) and F. nucleatum (species). This association appears to exist more in advanced stages of the tumour and/or adenoma and is often associated with worse prognosis and shorter survival.

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What do we already know about this topic?
CRC is one of the most common malignancies in the Western world. It is the fourth most common cause of cancer mortality worldwide, although screening programs based on fecal occult blood tests and subsequent colonoscopy have been shown to reduce mortality in patients who present with symptoms already developed. Most CRC is sporadic, resulting from chromosomal instability and dysplasia from adenomas to carcinomas

What is the main contribution to Evidence-Based Practice from this article?
There is a significant association between the intestinal microbiome and CRC, with emphasis on Fusobacterium and F. nucleatum. This association exists in advanced stages of the tumor and is often associated with worse prognosis and shorter survival

What are this research's implications towards health policy?
CRC remains a leading cause of morbidity and mortality, with a rising incidence expected to increase by 2030. The gut microbiome presents a new frontier in our understanding of CRC pathogenesis.

Authors' Contributions Statement:
Kennup: Conceptualization and Original Draft; Vianna: Formal analysis; Validation; Uyeda: Methodology; Gabriel::Visualization, Review and Editing

Introduction

Colorectal carcinoma (CRC) is one of the most common malignant tumours of the digestive tract, considered a major public health concern today (Sun et al., 2019). Overall, CRC ranks fourth in incidence (9.2% of total cancer cases) but second in terms of mortality (9.2% of total cancer deaths) (Sun et al., 2019). The malignant mechanism of CRC has not been fully established. The occurrence of CRC is a multifactorial and multistep process caused by the synergy of environment, diet, and lifestyle, along with genetic factors, while inflammation has been identified as an important risk factor (Sun et al., 2019).

CRC is considered one of the most common neoplasms in the Western world, with 1.2 million people diagnosed worldwide each year (Brenner, Kloor & Pox, 2014). It is the fourth most common cause of cancer mortality worldwide (Haggar & Boushey, 2009) and, although screening programs based on fecal occult blood tests and subsequent colonoscopy have been shown to reduce mortality by 16% (Towler et al., 1998) 70–90% of patients present with symptoms already developed (Moreno et al., 2016). In the United Kingdom, most patients are diagnosed at stage III–IV (52–56%) than at earlier stages I–II (44–48%) (Bowel, 2015) and the incidence in younger patients is increasing (Siegel et al., 2017). Five-year survival in the UK remains at 59% for men (Bowel, 2015) and 80% for women (Bowel, 2015).

Most CRCs are sporadic, resulting from chromosomal instability and dysplasia of adenomas to carcinomas (Strum, 2016), while hereditary familial adenomatous polyposis (FAP) and non-hereditary polyposis coli (HNPCC) syndromes arise due to germline APC gene mutations (Talseth-Palmer, 2017) and the microsatellite instability pathway (Chang, 2017). In addition, a serrated pathway thought to be centered on BRAF mutation and gene promoter hypermethylation has been described (Yamane, Scapulatempo-Neto, Reis & Guimarães, 2014).

The Gut Microbiome

The gut microbiome is known to play important roles in the function and well-being of human organs. Colonic bacteria are essential in the digestion of carbohydrates, proteins, and fatty acids (Fan et al., 2014). Mucosal epithelial barrier function (Malago, 2015) and tonic stimulation for systemic immunity (Littman & Pamer, 2011) are essential in the healthy formation of helper T cells and acquired immunity, and are achieved through an intricate balance between the host and the microbiome. It is profoundly sensitive to a wide range of factors and stressors induced by changes in diet (Singh et al., 2017), exercise (Monda et al., 2017), environment (Rothschild et al., 2018), oxygenation, and ischemia (Albenberg et al., 2014). Dietary changes over a period as short as 24 hours have been shown to reversibly alter the composition of the gut microbiome, with reversion back to baseline within 48 hours if the diet is not continued. Diets high in sugars have been shown to reduce the relative abundance of Bacteroides species in humans (Eid et al., 2014) and this has been reflected in type 2 diabetics who have an increased ratio of Firmicutes:Bacteroidetes.

Intestinal dysbiosis and colorectal cancer

Dysbiosis (a preponderance of pathogenic bacteria and a reduction in beneficial microbes) and associated chronic inflammation have previously been implicated in inflammatory bowel disease, irritable bowel syndrome, and type 2 diabetes mellitus (Jurjus et al., 2015) and are now known to facilitate carcinogenesis in CRC through genetic and epigenetic mechanisms (Borges-Canha et al., 2015).

The current bacterium of greatest interest in the intestinal dysbiosis of CRC is Fusobacterium nucleatum, a gram-negative anaerobe normally prevalent in the oral cavity (Gharbia, Shah, Lawson, & Haapasalo, 1990). In laboratory studies, F. nucleatum has been shown to increase tumour burden in mice (Kostic et al., 2013) even in the absence of a pre-existing model of colitis inflammation through the expression of pro-inflammatory markers and expression of virulence factors, such as adhesion to the FadA molecule on its epithelial surface to infiltrate the colon epithelium and activate the beta-catenin/WNT signalling pathway (Rubinstein et al., 2013), promoting carcinogenesis. In humans, F. nucleatum helps cancer cells build the tumour microenvironment and benefit from its resistance to chemotherapy. The constituent elements of the tumour environment, including neutrophils, macrophages, and lymphocytes, contribute to the existence of tumour cells, respectively (Luo et al., 2019). It has also been shown to play a role in modulating the immune response in human cells, causing T cell apoptosis by arresting the G1 cell cycle phase (Shenker & Datar, 1995) and expanding myeloid immune cells that inhibit the proliferation and functionality of clonal T cells (Nosho et al., 2016).

The most important mechanisms of Fusobacterium nucleatum involved in CRC carcinogenesis are immune modulation (such as the increase in myeloid-derived suppressor cells and natural killer cell inhibitory receptors), virulence factors (such as FadA and Fap2), microRNAs (such as miR-21), and bacterial metabolism (Hashemi et al., 2019).

Recent clinical studies have shown that F. nucleatum is enriched in several studies in colorectal adenomas and carcinomas; (Gao et al., 2015 Zhou et al., 2016) and gradually increases this progression, implicating its involvement in the adenoma-carcinoma sequence (Castellarin et al., 2012, Kostic et al., 2012), F. nucleatum has also been shown to be associated with high degrees of microsatellite instability-high CCR and CpG island methylator-like CCR (Tahara et al., 2014). Some

evidence has also emerged showing that F. nucleatum is associated with higher CCR staging and worse prognosis, (Flanagan et al., 2014, Wei et al., 2016) and plays a role in modulating chemoresistance in CCR (Yan et al., 2017). The enterotoxigenic molecular subtype of Bacteroides fragilis produces a toxin that drives the production of inflammatory mediators via the Wnt signalling pathway (Wu et al., 1998) inducing colitis in a murine model (Wick et al., 2014). In humans, there is evidence that it is associated with advanced CRC (Boleij, et al., 2015).

Peptrostreptococcus has been shown to increase colon cell proliferation and increase intracellular reactive oxygen species, leading to dysplasia in a murine model (Tsoi et al., 2017). It has also been shown to be enriched in tissue in CRC cases (Chen et al., 2012).

Porphyromonas species have also been associated with CRC, with oral bacteria found to be enriched in CRC (Ahn et al., 2013). The altered gut microbiome in CRC is also characterized by a reduction in certain bacterial taxa that are abundant in eubiosis with protective functions and properties; these include Bifidobacterium, Ruminococcus, and Facaelibacterium (Rivière et al., 2016). Faecalibacterium prausnitzii reduces NF-kB activity and reduces pro-inflammatory cytokines (Sokol et al., 2008). Genera such as Faecalibacterium have been shown to be depleted in clinical studies in CRC patients (Marchesi et al., 2011); depletion of such commensals by incomplete mechanisms is thought to allow the proliferation of the dysbiotic microbiome (Marchesi et al., 2011).

Surgery and intestinal dysbiosis

The iatrogenic effects of surgery also cause profound changes in the bacterial microbiome, the extent and significance of which remain incompletely understood (Kunzmann et al., 2019). Colectomy exposes segments of the intestine to oxygen and temporary ischemia via arterial ligation, which has been shown to alter the microbiome; some bacterial species have been shown to be protective in reducing subsequent mucosal injury in murine models (Wang et al., 2012).

In addition, the effects and clinical implications of our use of mechanical bowel preparation, fasting patients, and antibiotic use prior to surgery on the gut microbiome are not yet fully understood. Such measures were based on a traditional understanding of infectious prophylaxis; the goal was to decrease intestinal luminal content and bacterial burden. Mechanical bowel preparation instantly and dramatically changes the gut microbiome, with one study showing a 31-fold decrease in total microbial burden and taking approximately 14 days to recover to baseline (Jalanka et al., 2015). The combination of fasting, mechanical bowel preparation, and antibiotic administration, along with the physiological stress of ischemia and tissue oxygenation, may provide pathobionts with the opportunity to proliferate amidst the suppression of commensal species. Anatomical changes in anatomy following surgical resection are another consideration (Gralka et al., 2015). Increasingly, evidence is emerging for the importance of such changes in the gut microbiome in recovery and complications such as anastomotic leakage (Gaines, Shao, Hyman, & Alverdy, 2018).

Methods

A systematic review of the published literature was conducted in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines (Liberati et al., 2009). Literature searches were conducted on PubMed via the Advanced Search interface and EMBASE via the NHS

Healthcare Databases advanced search using MeSH terms, search terms and Boolean operators with synonyms plurals, and keywords. Studies with fewer than 10 participants with CRC, animal studies, case reports or series, abstracts or conference proceedings were excluded. Included studies had data extracted (if available) on the nature of the study: sample sizes of relevant patient groups (typically CRC, adenomatous disease and healthy controls), mean age of patients with CRC, any previous surgical treatment for CRC, the type of study sample (typically mucosal or stool samples) and the detection technique used. Studies were divided by bacterial taxon and then subdivided into those investigating mucosal tissue samples and stool samples. Mucosal tissue studies were then subdivided into those compared with healthy control groups or with matched adjacent normal tissue.

Pertinent associations of relevant gut bacteria with CRC or adenoma, as well as associations with tumour location, were noted. Depth of invasion, lymph node metastasis, distant metastasis, patient age, overall staging, and finally any prognostic or predictive implications were also noted. All included articles were assessed for methodological quality using a modified Cochrane Collaboration tool to assess the risk of bias (Higgins, 2011).

Results:

A PRISMA diagram is shown below (Figure 1), where the inclusion and exclusion criteria were applied and resulted in 53 relevant studies for inclusion in this review, with a total of 5167 CRC patients, of which 3754 were tested on mucosal tissue samples, 1072 on stool samples and 341 in a combination (mean ages 65.66, 61.74 and 68.78 based on available data). No randomised controlled trials were found, with the vast majority being cohort or case-control studies. 16rRNA and quantitative PCR were the most common microbiome analysis techniques. The demographics and results of the ten most relevant studies are summarised in Table 1.

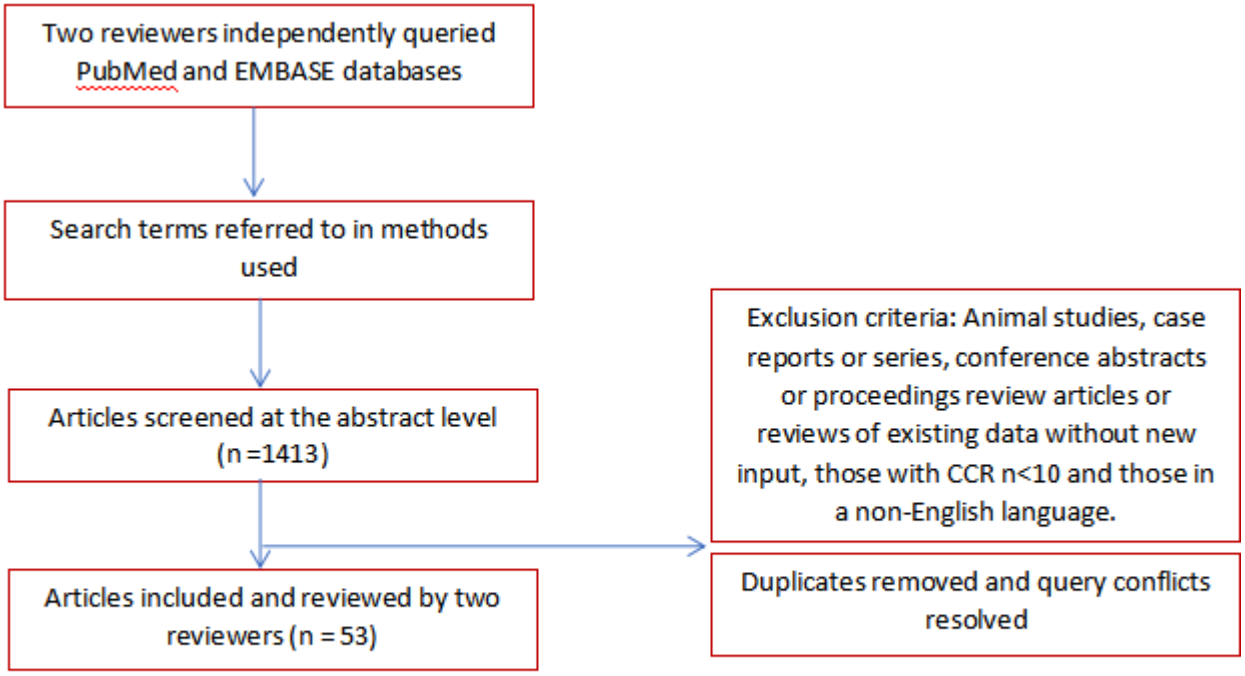

Figure 1: PRISMA diagram

Authors, study design, nationality, and year	Sample size and information	Mean patient age with CCR (years)	Specimen type and detection method	Associations with colorectal neoplasia	CCR location	CCR depth of invasion	CCR lymph node metastasis	CCR distant metastasis	CCR Staging and Prognosis
Amitay *et al.* Cohort study Germany 2017	CCR n=50 Advanced adenoma (TV or villous, HGD or ⩾1cm) n=113 Nonadvanced adenoma n=110 Healthy controls n=231 Examining role of *Fusobacteria* in CCR.	66.9	Stool 16rRNA qPCR	**Genus** *Fusobacterium* more associated with CCR than advanced adenoma, nonadvanced adenoma and controls (p<0.001). Not more significantly associated with any adenoma vs. controls **Species and subspecies** *F.nucleatum* more common in CCR vs. all other groups (p<0.022). *F.periodonticum* subspecies significantly increased in CCR vs. controls (p=0.003).	-	-	-	-	*F.nucleatum* relative abundance positvely associated with advanced CCR stage (stage I vs. II p=0.012, and stage I vs. III p=0.042) Predictive model using *F.nucleatum* for CCR vs. rest of study population AUC=0.676.

Gao *et al.* China 2015 Cohort Study	CCR n=31 Healthy controls n=30 Adjacent tumour tissue also. Examining gut microbiota and CCR.	67±7.2	Mucosal tissue 16rRNA PCR	**Phylum** Firmicutes ($p<0.001$), and Fusobacteria ($p<0.001$) significantly increased in CCR vs. normal controls, with Proteobacteria ($p<0.001$) significantly decreased. Firmicutes significantly increased between tumour and tumour-adjacent normal tissue ($p=0.03$) and Proteobacteria decreased ($p<0.01$). Fusobacteria not significantly different between tumour and adjacent tissue. **Genus** *Lactococcus* ($p<0.007$), *Fusobacterium* ($p=0.032$), *Escherichia-Shigella* ($p=0.004$) and *Peptostreptococcus* ($p=0.004$) were	-	-	-	-	-

				significantly enriched in CCR vs. normal controls. *Fusobacterium* not significantly different in CCR vs. adjacent normal tissues.					
Kostic *et al.* Spanish, USA, Vietnam 2022 Cohort study	CCR n=95 Matched adjacent normal tissue pairs. Examining *Fusobacterium* and CCR.	-	Mucosal tissue Whole genome sequencing 16rRNA qPCR	**Genus** *Fusobacterium* more abundant in CCR compared to normal matched tissue (p=0.0003).	No association between *Fusobacterium* and location.	-	-	-	-
Kostic *et al.* UK, USA 2023 Cohort study	CCR n=27 Adenomas n=28 Normal n=31 Matched adjacent normal tissue pairs. Examining association between Fusobacteria and adenomas and CCR.	-	Mucosal tissue and stool 16rRNA qPCR FISH	**Phylum** *Fusobacteria* significantly more abundant in CCR stool samples versus adenomas (p<0.0005) and normal (p>0.0005) samples. *Fusobacteria* significantly more abundant in adenoma vs. matched normal tissue (p<0.004).	-	-	-	-	-

Nakatsu *et al.* China 2015 Cohort study	CCR n=52 Adenoma n=47 Normal controls n=61 Additional Chinese cohort: Normal control, n=25, adenoma n=41, adenocarcinoma n=50 Paired adjacent samples. Examining association between gut microbiota and CCR	67.85±13.18	Mucosal tissue Metagenomics 16rRNA PCR	5 meta-communities identified. Metacommunity E, enriched in *Fusobacterium* and other periodontal/oral microbiota strongly associated with adenomas and carcinomas (q<1x10-5). **Genus** *Gemella* (q<0.0001) *Parvimonas* (q<0.05), *Peptostreptococcus* (q<0.0001) and *Granullcatella* (q<0.0001) all significantly enriched in CCR vs normal controls. All also significantly enriched vs. adenoma-adjacent, adenoma and carcinoma-adjacentissue. **Species** *B. fragilis* significantly enriched in CCR vs. normal tissue (q<0.0001),	-	-	-	-	-

				adenoma-adjacent (q<0.0001), adenoma (q<0.001) and carcinoma-adjacent (q<0.01) tissues.					
Wei *et al.* China 2016 Cohort study	CCR n=180 Survival group (lived more than 3 years without signs of recurrence) n=92 Non-survival group (died within 3 years for CCR related causes) n=28 Recurrence group (recurrence or metastasis within 3 years but survived) n=31 Unclear n=29 Tumour tissue and adjacent normal tissue Examining the role of gut microbiota in CCR, and association with survival	62.2	Mucosal tissue Immunohistochemistry 16rRNA PCR	Survival group more abundant than non-survival *Shewanella* (FDR=0.091), *Methylobacterium* (FDR=0.039), *Faecalibacterium* (FDR=0.016), *Sphingomonas* (FDR=0.031), *F.prausnitzii* (FDR=0.028), *Methylobacterium suomiense* (FDR=0.098) Survival group more abundant than the recurrence group: *Methylobacterium* (FDR=0.09), *Mycoplasma* (FDR=0.01) Non-survival group more abundant than the survival group: *B.fragilis* (FDR=0.017)	-	High abundance of *F.prausnitzii* (p=0.015) and *F.nucleatum* (p=0.015) significantly associated with depth of invasion.	High abundance of *F.nucleatum* significantly associated with lymph node metastasis (p=0.011)	-	High abundance of *B.fragilis* (p=0.001) and *F.nucleatum* (p=0.003)associated with worse 3 year OS, and worse DFS (p<0.001 and p=0.001) than low abundance. High *F.nucleatum* and *B.fragilis* independent predictors of 3-year OS and DFS.

				Recurrence group more abundant than the survival group: *F.nucleatum* (FDR=0.08)					
Yan *et al.* China 2017 Cohort study	CCR stage III/IV n=280 pairs tumour and tumour adjacent Examining *F.nucleatum* in advanced CCR	-	Mucosal tissue qPCR Immunohistochemistry	**Species** *F.nucleatum* level significantly higher in CCR vs. adjacent normal tissue (p<0.001)	No association between *F.nucleatum* level and tumour location.	*F.nucleatum* significantly associated with tumour invasion (p=0.015)	*F.nucleatum* significantly associated with positive lymph node status (p=0.008)	*F.nucleatum* significantly associated with distant metastasis (p=0.020)	*F.nucleatum* also significantly higher in stage III (p<0.001) and stage IV (p=0.005) vs. adjacent normal tissue. High *F.nucleatum* level associated with significantly worse cancer-specific survival (CSS) (p<0.001) and disease-free (DFS) survival (p<0.001) *F.nucleatum* level indepdent risk factor for both CSS (p<0.001) and DFS (p<0.001) High *F.nucleatum* level in stage IIIb significantly associated with worse CSS (p=0.038) and DFS (p=0.029), and in stage IIIc (CSS p=0.035), DFS p=0.048) stage IV also (CSS p=0.042, DFS p=0.019) Adjuvant chemotherapy (AC) treatment was associated with a significantly better clinical outcome in both patients with low *F. nucleatum* level (CSS: p<0.001, DFS: p<0.001) and high *F. nucleatum* level (CSS: (p=0.034, DFS: p=0.024) Patients with low *F.nucleatum* levels

									benefit more from AC vs. high (DFS p=0.048).
Yu *et. Al* China 2015 (Cohort study) 2017 [65] (Cohort study)	Two studies. **Initial Study**: Normal controls n=52 Advanced adenomas n=47 CCR=42 Examining prevalence of different bacteria in the above groups. **Repeat study** re-examined some of the above data with additional cohorts looking at patients with CCR recurrence. 3 Cohorts examined: Cohort 1: Recurrent CCR n=16 Non-recurrent CCR n=15 Cohort 2: Recurrent CCR n=44 Non-recurrent CCR n=48 Cohort 3: Recurrent CCR n=87	-	Stool 16rRNA and qPCR	**Genus and species** Initial study: Significantly tended to increase in relative abundance from normal tissue to adenomas to CCR: *Fusobacteria* (p=0.031), (*Escherichia/Shigella)* : (p=0.025), *Coprococcus* (p=0.034), *Streptococcus* (p=0.016), *Enterococcus* (0.004) Significantly tended to decrease in relative abundance from normal tissue to adenomas to CCR - *Actinobacteria: Actinomyces* (p<0.001), *Bifidobacteria*: (p=0.008), *Firmicutes: Blautia* (p<0.001), *Clostridium* (p<0.001), *Dorea* (p<0.001), *Lactobacillus*		Cohort 2) High *F.nucleatum* not significantly associated with depth of tumour invasion (to serosa).	-	-	(Cohort 2) High *F.nucleatum* significantly associated with AJCC staging III (p=0.022) and tumour size >15cm^3 (p=0.018) (Cohort 2) High amount of *F.nucleatum* strongly associated with shorter recurrence-free survival. (logrank p=6e^{-06}) Cohort 3) High amount of *F.nucleatum* strongly associated with shorter recurrence-free survival. (logrank p=6.4e^{-10}) 5-year recurrence survival substantially shorter in *F.nucleatum* high vs. low group. (Cohort 2) *F.nucleatum* based receiver operator characteristic curve analysis to predict CCR recurrence significantly superior to AJCC-based model (0.776 vs. 0.646, p=0.039)

	Non-recurrent CCR n=86			(p=0.011), *Roseburia* (p=0.003), *Eubcaterium* (p=0.013). Subsequent study: re-analysed data looking at recurrence: (Cohort 1) *F.nucleatum* (p<0.01), *P. micra* (p<0.05) and *P. anaerobius* (p<0.05) more abundant in recurrent CCR vs. non-recurrent, with *F. nucleatum* most abundant in quantity. (Cohort 2) *F.nucleatum* more abundant in recurrent vs. non-recurrence (p<0.01). *F.nucleatum* also more abundant in CCR adjacent tissues in both recurrent (p<0.05) and non-recurrent CCR (p<0.05)					

				(Cohort 3) *F.nucleatum* more abundant in quantity recurrent vs. non-recurrence ($p<0.01$) High *F.nucleatum* abundance significantly associated with CCR recurrence (73.4% vs. 30.9%, $p=2.436e^{-8}$)					
Zackular *et al.* USA 2024 Cohort study	CCR n=30 Adenoma n=30 Healthy controls n=30	59.4	Stool 16rRNA PCR	**Genus** *Fusobacterium* ($p=0.001$), *Porphyromonas* ($p=0.002$), *Enterobacteriaceae* ($p=0.040$) significantly enriched in CCR vs. normal controls. *Bacteroides* ($p=0.008$) significantly decreased in CCR vs. controls. *Fusobacteria* significantly ($p<0.001$) enriched	*Fusobacterium* relative abundance not significantly associated with tumour location.	-	-	-	*Fusobacterium* relative abundance not significantly associated with tumour stage Combining microbiome OTUs with the model for prediction of CCR based on age, BMI and race improved AUC from 0.798 to 0.922

				in CCR vs. adenomas.					
Zeller *et al.* France, Germany, Denmark, Spain 2024 Cohort study	3 study populations: France: CCR n=55, adenoma n=42, healthy n=61 Germany: CCR n=38, Population H: healthy n=297 Matched adjacent tissue samples	67.8	Stool and mucosal tissue 16rRNA PCR	**Species** Significantly increased in CCR vs normal tissue: *Fusobacterium nucleatum* subsp. *nucleatum* (p=6.54e−04), *Fusobacterium nucleatum* subsp. *animalis* (p=7.51e−05), *Fusobacterium nucleatum* subsp. *vincentii* (p= 1.30e−05), *Fusobacterium nucleatum* subsp. *polymorphum* (p=3.23e−03), *Pseudoflavonifractor capillosus* (p= 1.07e−03), *Porphyromonas asaccharolytica* (p= 9.61e−03), *Prevotella nigrescens* (p=2.15e−02), *Peptostreptococcus stomatis* (p=2.20e−02), *Leptotrichia hofstadii* (p=3.50e−02), *Parvimonas*	-	-	-	-	Significantly enriched in early stage CCR (0/I/II/) vs tumour-free: Significantly enriched in early stage CCR (0/I/II/) vs tumour-free: *F. nucleatum subsp. vincentii* (p<1E-5), *F. nucleatum subsp. animalis* (p<1E-5, *Peptostreptococcus stomatis* (p<0.05, *Porphyromonas asaccharolytica* (p<0.05).

				micra (p=6.65E−02), *Bacteroides fragilis* (p=7.46e−02), *Bilophila wadsworthia* (p=8.41e−02),					

Table 2: Relevant studies identified from the systematic review. Abbreviations: AJCC – American Joint Committee on Cancer staging, AL – anastomotic leak, ANCOM- analysis of composition of microbiomes, AUC – area under curve, AUROC – area under the receiver operator curve, bft – *Bacteroides fragilis* toxin, BMI – body mass index, CMS - consensus molecular subtype, CCR – colorectal cancer, DFS – disease free survival, ETBF – enterotoxigenic *Bacteroides fragilis;* FDR – false discovery rate, FISH – fluorescence in-situ hybridisation, HGD – high grade dysplasia, HP – hyperplastic polyp, MSI-H – microsatellite instability high, OS – overall survival, OTU – operational taxonomic unit, pan-DSRA - dissimilatory sulfate reductase, PCR – polymerase chain reaction, qPCR – quantitative polymerase chain reaction, PER-MANOVA – permutational multivariate analysis of variance, rRNA – ribosomal ribonucleic acid, SSA – serrated sessile adenoma, TA – tubular adenoma, TNM – tumour nodal metastases staging, TSA – traditional sessile adenoma, TV – tubulovillous adenoma.

Fusobacterium nucleatum (F. nucleatum) and its genus Fusobacterium (Phylum: Fusobacteria, Order: Fusobacteriales, Family: Fusobacteriaceae) were the most commonly investigated taxa in the CRC microbiome. All studies used a minimum of RNA sequencing (usually using a 16rRNA amplicon sequencing method) and eight studies complemented this with PCR. Kostic et al. (Kostic et al., 2013, Kostic et al., 2012) additionally used whole genome sequencing and FISH techniques in their two studies, respectively.

For 219 CRC patients, the presence or abundance of Fusobacterium was compared in healthy control groups. CRC patients were compared with matched adjacent normal tissue biopsied from the same patients. A total of 656 CRC patients and 634 CRC samples were examined.

Regarding the Fusobacterium genus in CRC mucosal tissue versus healthy control groups, 219 CRC patients showed a significant positive association ($p < 0.05$) between Fusobacterium and CRC. Flemer et al. (Flemer et al., 2017) identified an increased abundance of OTUs belonging to several genera, particularly those reported as oral pathogens, including Fusobacterium. Gao et al. (Gao et al., 2015) identified that the CRC microbiome was significantly different from that of controls, with a significantly increased level of Fusobacteria and Firmicutes at the phylum level ($p < 0.001$); Fusobacterium at the genus level was also significantly enriched ($p = 0.032$). Regarding Fusobacterium genera in CRC mucosal tissue versus matched adjacent non-neoplastic tissue, a total of 437 CRC patients were analyzed and showed a significant positive association between Fusobacterium in tumour versus adjacent samples. Allali et al. (Allali et al., 2015) compared tumour samples from two cohorts from the USA and Spain (total of 45 CRC patients) and found that Fusobacterium was significantly increased in the Spanish cohort ($n = 23$, $p < 0.0001$) and this result was also found by Burns et al (Burns et al., 2015). Kostic et al. (Higgins, 2011) found that Fusobacterium is significantly positively associated in 95 CRC samples vs. matched tissue; qPCR confirmed a significantly increased abundance ($p < 0.0003$); This was in agreement with whole genome sequencing, which found that Fusobacterium sequences were significantly enriched in colorectal cancer metagenomes. Subsequent 16rDNA FISH visualized Fusobacterium expression in CRC cells that were again enriched versus matched non-neoplastic tissue (Higgins, 2011). Nakatsu et al. (Nakatsu et al., 2015) used a modelling approach based on 16rRNA sequencing and Dirichlet multinomial mixture models to identify five distinct groups of metacommunity bacteria. Metacommunity E comprising Fusobacterium and other oral and pathogenic bacteria was significantly associated ($q < 1.0x10-5$) with CRC and adenomas; overall, 40% of adenomas and 48% of all CRCs were classified as belonging to this group (Nakatsu et al., 2015).

Regarding the Fusobacterium genus in stool samples from CRC versus healthy controls and for 326 CRC patients, the presence of Fusobacterium abundance was compared relative to healthy control groups. The results showed a significant positive association between Fusobacterium and CRC. Regarding the Fusobacterium genus in adenomas: mucosal tissue versus healthy controls or matched adjacent tissue, a significant positive association was observed. Kostic et al. (Kostic et al., 2013) found Fusobacterium enriched ($p < 0.004$) in 48% of adenomas ($n = 29$) versus adjacent normal tissue. Nakatsu et al. (Nakatsu et al., 2015) found that the metacommunity including Fusobacterium and similar oral pathogens was significantly associated with

adenomas and carcinomas (q < 1.0x10-5) compared to healthy controls. Xu et al. (Xu & Jiang, 2017) found no significant difference between adenomas and normal groups.
Regarding Fusobacterium genus in adenomas: stool samples versus healthy controls, a total of 328 CRC patients were evaluated to verify the existence of a significant difference between Fusobacterium in adenoma stool samples and healthy controls. Kostic et al. (Kostic et al., 2013) (n = 28) found a significant increase ($p < 5 \times 10-3$) compared to healthy controls. Yu et al. (Yu et al., 2015) (n = 47) also found a significant increase and interestingly observed a significant progression in Fusobacterium abundance from normal adenomatous disease to carcinomatous disease (p = 0.034). Amitay et al. (Amitay et al., 2017) (n = 223) found no association between normal controls and any type of adenoma (non-advanced adenoma n = 110, advanced adenoma, i.e., tubulovillous, villous, or high-grade dysplasia ⩾1cm), as did Zackular et al (Zackular et al, 2011) (n = 30).
Regarding the Fusobacterium genus in mucosal tissue or stool from CRC versus adenomas, Amitay et al. (Amitay et al., 2017) found Fusobacteria (q < 0.001) at the phylum and Fusobacterium (p < 0.01) at the genus level to be significantly enriched in CRC vs. adenomas. Zackular et al. (Zackular et al, 2011) found that the OTU assigned to Fusobacterium had a higher relative abundance (p < 0.001) in CRC vs. adenomas.
In Zackular et al. (Zackular et al, 2011) (30 CRC, adenomas, and healthy patients), age, race, and BMI were predictive of carcinomas (AUC = 0.798; 95% CI: 0.686–0.910; p < 0.001) in feces vs. normal controls. Amitay et al. (Amitay et al., 2017) (44 CRC patients, 223 adenomas, 193 healthy controls) showed that a predictive model based solely on Fusobacterium for CRC vs. the rest of the study population had an AUC of 0.676, which increased to 0.715 when combined with age and sex. Fusobacterium was not found to improve prediction between advanced or non-advanced adenomas and healthy controls.
In studies, 1532 CRC patients and 1031 samples found a significant positive association between F. nucleatum and CRC versus matched adjacent normal tissue.
Regarding F. nucleatum in CRC stool samples versus healthy controls, 433 CRC patients and 275 samples showed a significant positive association between F. nucleatum and CRC. Liang et al. (Liang et al., 2016) showed that F. nucleatum is significantly more abundant in CRC based on metagenomics, confirmed by qPCR controls (P < 0.0001), with predictive value as well (AUROC of 0.868 (P < 0.0001)).
Regarding F. nucleatum species in mucosal tissue or adenomatous stool samples vs. normal tissue or healthy controls, a total of 115 CRC patients had their mucosal tissue studied and found that F. nucleatum was significantly enriched in adenomas versus normal tissue. Flanagan et al. (Flanagan et al., 2015) (n = 52) found that F. nucleatum was not significantly more abundant in PCR vs. matched normal adjacent tissue for all adenomas, but significantly enriched in high-grade dysplasia (p = 0.015). McCoy et al. (McCoy et al., 2013) (n = 67) also found an increased abundance of F. nucleatum compared to controls (p = 0.01). Individuals with high F. nucleatum (compared to the lowest tertile) were also significantly more likely to have adenomas (OR 3.66, 95% CI 1.37–9.74, p = 0.005). Regarding F. nucleatum species in mucosal tissue or stool samples from CRC vs. adenomas, Ito et al. examined F. nucleatum expression in hyperplastic polyps (n = 129), sessile serrated adenomas (n = 120), traditional sessile adenoma (n = 94), nonserrated adenomas (n = 122), and CRCs (n = 511). F. nucleatum was positive in 24% of

HPs, 35% of SSAs, 30% of TSAs, 33% of nonserrated adenomas, and 56% of CRCs. F. nucleatum positivity was found significantly more frequently in CRC than in all groups ($p < 0.0001$).

Regarding F. nucleatum in mucosal tissue and CRC staging, a total of 1784 CRC patients were studied and a significant positive association was found between F. nucleatum and an element of CRC staging. Mima et al. (Mima et al., 2016) examining 1069 CRC patients found that F. nucleatum is significantly associated with higher pT staging ($p = 0.0003$ and $p = 0.0007$ in univariate and multivariate analyses), but not with pN or M stages. Similarly, Wei et al. (Wei et al., 2015) studied 180 patients with RCC and found that a high abundance of F. nucleatum was associated with increased depth of tumour invasion ($p = 0.015$) and lymph node metastasis ($p = 0.011$). Yan et al. (Yan et al., 2017), who studied 280 patients with stage III/IV RCC, found significantly higher F. nucleatum in stages III ($p < 0.001$) and IV ($p = 0.005$) versus adjacent normal tissue.

Mima et al. (Mima et al., 2016) found that F. nucleatum positivity was associated with worse cancer-specific survival (compared with negative cases, multivariable hazard ratios (HRs) for cancer-specific mortality in F. nucleatum-low and F. nucleatum-high cases were 1.25 (95% confidence interval [CI], 0.82–1.92) and 1.58 (95% CI, 1.04–2.39), respectively. High F. nucleatum abundance was also associated with poorer cancer-specific survival, $p = 0.023$). Wei et al. (Wei et al., 2016) found an association with worse 3-year overall survival ($p = 0.003$) and disease-free survival ($p = 0.001$).

Yan et al. (Yan et al., 2018) found that a high F. nucleatum level was associated with significantly worse cancer-specific survival ($p < 0.001$) and disease-free survival (DFS) ($p < 0.001$), and was an independent risk factor for both. Treatment with adjuvant chemotherapy with CSS ($p < 0.001$) and DFS ($p < 0.001$) was associated with a significantly better clinical outcome in both patients with low F. nucleatum level (CSS: $p < 0.001$, DFS: $p < 0.001$) and high F. nucleatum level (CSS: ($p = 0.034$, DFS: $p = 0.024$), but patients with low F. nucleatum levels benefit more from CA vs. high (DFS $p = 0.048$).

Regarding F. nucleatum in feces and CRC; staging and prognosis, 185 CRC patients were investigated and a significant positive association between F. nucleatum and staging was found. Yu et al. (Yu et al., 2017) (44 CRC patients examined) found F. nucleatum significantly associated with AJCC III staging ($p = 0.022$) and tumour size > 15cm3 ($p = 0.018$). Receiver operator characteristic curve analysis based on F. nucleatum to predict CRC recurrence significantly superior to the AJCC-based model (0.776 vs. 0.646, $p = 0.039$) and a high amount of F. nucleatum strongly associated with shorter recurrence-free survival (logrank p = 6e-06).

Zeller et al. (Zeller et al., 2014) found several F. nucleatum subspecies to be enriched in early-stage CRC (0/I/II); F. nucleatum subsp. vincentii ($p < 1e-5$) F. nucleatum subsp. animalis ($p < 1e-5$) vs. tumour-free samples.

Discussion

CRC remains a leading cause of morbidity and mortality, with an increasing incidence expected to increase by more than 60% by 2030. (Arnold et al., 2017) According to the American Cancer Society, approximately 147,950 new cases of CRC were estimated for the year 2020, with approximately 53,200 deaths among Americans (American...). The intestinal microbiome presents a new frontier in our understanding of the pathogenesis of colorectal cancer, and we are increasingly understanding that altering elements of the

intestinal microbiome offers us new opportunities in our detection, diagnosis, staging, investigation and treatment of CRC. Given the limitations in our current screening modalities, understanding and establishing an association between the intestinal microbiome and CRC may be the first step in its use as a biomarker. Our systematic review showed that there is indeed a significant association between many different taxa of the gut microbiome, including most notably Fusobacterium (genus) and Fusobacterium nucleatum (species), and CRC. This is consistent with the systematic review published by Hussan et al. (Hussan et al., 2017). All six studies examining mucosal samples of the genus Fusobacterium versus healthy controls and 8 of 9 studies examining stool samples found that Fusobacterium is significantly enriched in CRC. Similarly, all ten studies examining F. nucleatum in mucosal tissue and all four studies examining stool samples found that F. nucleatum is significantly enriched in CRC. Our study also found that other Fusobacterium species, including F. periodonoticum (Amitay et al., 2017) and Fusobacterium oral taxon 370, (Feng et al., 2015) and at the subspecies level F. nucleatum subsp. vincentii and F. nucleatum subsp. animalis (Zeller et al., 2014) were significantly enriched in CRC. This project also highlighted an important link between Fusobacterium and advanced tumour stage and/or poor long-term survival. The association of Fusobacterium and CRC can be explained at the molecular and histopathological levels, as this is in line with our existing knowledge of Fusobacterium, which has been shown to infiltrate epithelial cells, express pro-inflammatory markers (Kostic et al., 2013) and modulate the immune response (Nosho et al., 2016). Dejea et al. identified bacterial biofilms demonstrating bacterial invasion into the tumour mass (Dejea et al., 2014). Furthermore, the association between Fusobacterium and advanced tumour stage can be explained by the recent theory that is gaining evidence on the role of oral bacteria (such as Fusobacterium) in colorectal cancer, forming biofilms, and driving pathogenesis. Flemer et al. (Flemer et al., 2018) identified similar bacterial networks in oral swabs and colonic mucosa, both inside and outside the tumour; the addition of oral microbiota to those identified in stool also improved the prediction of CRC and adenomas.

The most consistent findings of Fusobacterium and F. nucleatum enrichment came from mucosal studies; although consensus showed significance even in stool samples. Variations in stool sample extraction and preservation methods exist, and other confounding factors, including the use of bowel preparation, make the modality less robust for detecting tumour-associated gut microbiomes.

Significant enrichment of Fusobacterium (genus) was observed in two of three mucosal studies and two of four stool studies in colorectal adenomas versus tissue or healthy controls. F. nucleatum (species) was similarly mixed, with two of four studies showing a significant difference in adenomas versus tissue or healthy controls. This may be related to the reliability of stool sampling as a modality, or it may be consistent with the observation that Fusobacterium is more strongly associated with disease progression along the adenoma-carcinoma sequence; (Yu et al., 2017) we were unable to stratify pooled results from identified studies due to heterogeneity in specification and inclusion of adenomas.

However, we observed a strong enrichment in CRC versus adenomas, in 3 of 3 Fusobacterium (genus) and 5 of 5 F. nucleatum (species); all Fusobacterium studies were mucosal and F. nucleatum studies were a mixture of mucosal

and fecal samples. This significant difference supports Fusobacterium and Fusobacterium nucleatum being pathogenic in the development of adenomas to CRC and again later in the adenoma-carcinoma sequence.
We did not find a consensus on gut microbiota and tumour location. However, the largest study to investigate this issue was Mima et al. (Mima et al., 2016) in a North American cohort of 1069 patients, who found a significant association between higher F. nucleatum levels and more proximal tumours ($p < 0.001$), with linear progression from the rectum to the cecum along the colon.
A strong trend in the evidence pointed to a significant association between F. nucleatum and advanced stage CRC, worsening prognosis and shorter survival. Four of the five mucosal studies that examined prognosis found a significant positive association with F. nucleatum, producing a worse prognosis for patients with CRC, and both stool studies (185 patients with CRC) found a significant positive association between F. nucleatum and staging. Discrepancies may exist due to heterogeneity in staging reports, with studies reporting elements of the TNM, Duke, and AJCC systems. This association with higher staging and subsequent worse prognosis is consistent with laboratory evidence and the theory that F. nucleatum is pathogenetically implicated in more advanced adenomas and CRC.
No studies identified have investigated the association of the gut microbiome with surgical resection or intervention in CRC, and this is an area of research that requires further study.

While the scope of this study provides a strong overview of the gut microbiota association, there are several limitations to the study. The absence of a meta-analysis due to the heterogeneity of many of the studies present will limit the scope of some of our conclusions. There were variations in testing methods, stage, and tumour location. Furthermore, all of the published research was performed in patients with established diseases (CRC or adenomas) with no population-based research to validate any of the prediction models created by some of the studies.
Finally, there is very little evidence of the impact of surgery on the microbiome and further research is needed. There are ongoing studies examining the role of the gut microbiome in anastomotic leakage and the results are awaited (ISRCTN, 2018).

Conclusion

There is a significant association between the gut microbiome and CRC, with emphasis on Fusobacterium (genus) and F. nucleatum (species). This association appears to be more prevalent in advanced stages of the tumour and/or adenoma and is often associated with worse prognosis and shorter survival.
Several microbiome-enhanced diagnostic predictive models exist, but further prospective validation is needed before they can be recommended for use in clinical practice. There is promising but limited research on the impact of the gut microbiome and therapy, but more research is needed in this area.

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