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The intestinal microbiota in colorectal cancer metastasis – Passive observer or key player?

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ABSTRACT

The association between colorectal cancer (CRC) and alterations in intestinal microbiota has been demonstrated by several studies, and there is increasing evidence that bacteria are an important component of the tumour microenvironment. Bacteria may contribute to the development of CRC metastasis by signalling through metabolites, promoting epithelial-mesenchymal transition, creating an immunosuppressive microenvironment and through the impairment of the gut-vascular barrier. Host immunity and intestinal microbiome symbiosis play a key role in determining innate and adaptive immune responses at the local and systemic level. How this gut-systemic axis might contribute to the development of CRC metastasis is however unclear. Several clinical trials are investigating the impact of microbiome-targeted interventions on the systemic inflammatory response, treatment-related complications, and side effects. This review examines pre-clinical and clinical studies which have examined the role of microbes in relation to CRC metastasis, the mechanisms which may contribute to tumour dissemination, and directions for future work.

1. Introduction

According to the World Health Organisation GLOBOCAN database, colorectal cancer (CRC) accounts for 10% of annually diagnosed cancers globally and is the second leading cause of cancer-related death worldwide (Globocan, 2020). Approximately 25% of patients will have metastatic disease at diagnosis with a five-year survival of 10% (CRUK, 2021). The most common metastatic sites include the liver, lung and peritoneum. Approximately 20–30% of patients will present with colorectal liver metastasis at the time of diagnosis or develop liver metastasis after initial surgery to remove the primary tumour (Manfredi et al., 2006; Hackl et al., 2014). Approximately 5–8% of patients will present with lung-only metastasis (Siebenhüner et al., 2020; Ge et al., 2020). Peritoneal metastases are present in 5–10% of patients at the time of primary surgery, in 4–19% during postoperative surveillance and in 40–80% of patients who die as a result of CRC (Koppe et al., 2006; Segelman et al., 2012).

The term microbiome refers to the collective microorganisms (microbiota) found in a particular environment. For example, the gut microbiome refers to all microorganisms (bacteria, virus, fungi) found in

Abbreviations: CAM, Cell adhesion molecule; CARD3, Caspase activation and recruitment domain 3; CRC, Colorectal cancer; CTLA-4, Cytotoxic T lymphocyte-associated antigen 4; CXCL1C-X-C, Motif Chemokine Ligand 1; CXCR6, C-X-C Motif Chemokine Receptor 6; ECM, Extracellular matrix; FMT, Faecal microbiota transplantation; GVB, Gut vascular barrier; HER2, Human epidermal growth factor receptor 2; IL-8, Interleukin-8; KRAS, Kirsten rat sarcoma; LPS, Lipolysaccharide; mAb, Monoclonal antibody; MAMP, Microorganism-associated molecular pattern; MMP, Matrix metalloproteinase; MMR, Mismatch repair; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NKT, Natural killer cells; MSI, Microsatellite instability; PD-1, Programmed cell death protein 1; PD-L1, Programmed cell death ligand 1; PV-1, Plasmalemma vesicle-associated protein 1; SCFA, Short-chain fatty acids; TLR, Toll-like receptor; u-PA, Urokinase plasminogen activator; VEGF-C, Vascular endothelial growth factor C; VEGFR, Vascular endothelial growth factor receptor.

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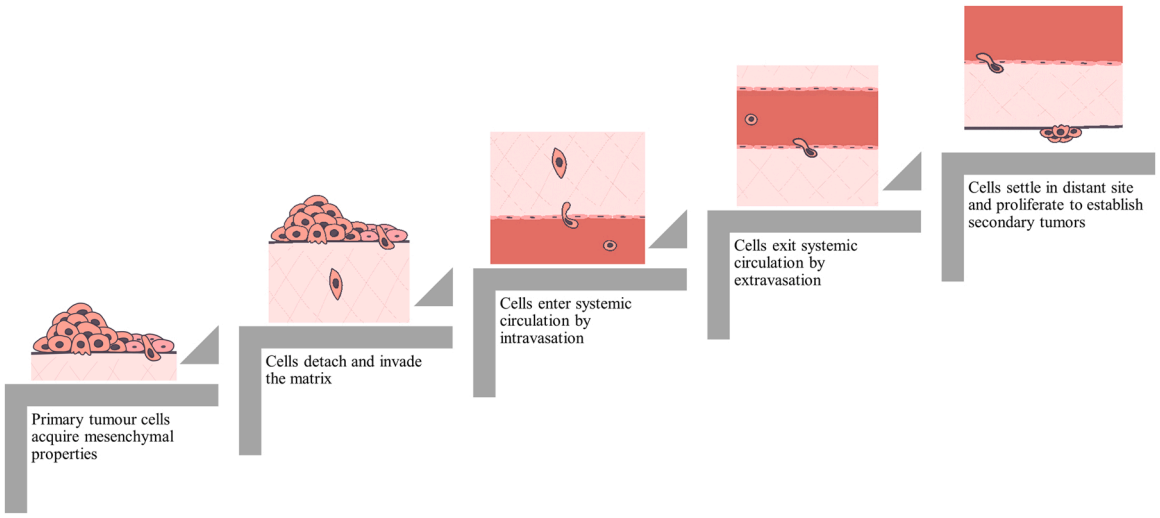

Fig. 1. Stepwise process of tumour metastasis (Obenauf and Massague, 2015). Figure created using Adobe Fresco (Version 3.6.2).

the gastrointestinal tract. It is now well-established that the intestinal microbiota are not merely commensals but have a mutualistic relationship with the host (Thursby and Juge, 2017). The majority of studies investigating the gut microbiome focus on the large intestine.

Adverse changes in the composition or activities of host-associated microbiota are often referred to as dysbiosis. For the intestinal microbiome this has been associated with several disease states including cancer. Whilst there is no universally agreed definition for the term dysbiosis, it is frequently defined as a reduction in microbial diversity or an imbalance in microbial taxa within the gastrointestinal tract. Unsurprisingly, CRC is associated with alterations in intestinal microbiota across various stages of tumour development (Feng et al., 2015). Whilst numerous studies have reported on the role of microbes in the development of primary CRC, their role in the progression to metastatic CRC is only beginning to be understood. This review aims to summarise preclinical and clinical studies which have examined the role of bacteria in the development of CRC metastasis and outline areas for future work.

2. Search strategies

Pub med and Ovid MEDLINE were used to identify relevant publications (Supplementary Figure 1). Search terms for Pub med include "colorectal cancer" and "bacteria or microbiome" and "metastasis or metastases". Search terms for Ovid MEDLINE include "colorectal cancer or colorectal neoplasms" AND "metastasis" or "metastases" or "metastatic" AND "bacteria" or "microbes" or "microbiome". Exclusion criteria were unrelated, duplicated, unavailable full texts or abstract-only papers. Inclusion criteria were pre-clinical and translational articles relevant to the review. Clinical trials with microbiome-targeted interventions in patients with CRC were identified from clinicaltrials.gov (Supplementary Figure 2).

3. Biological overview of metastasis

Tumour metastasis develops through a sequential stepwise process that is illustrated by Fig. 1. This begins with the detachment of cells that have acquired mesenchymal properties from the primary tumour, invasion into the surrounding matrix, intravasation of tumour cells into the systemic circulation, extravasation of tumour cells into distant tissues, the proliferation of tumour cells within extra-colonic locations and establishment of macroscopic tumours (Obenauf and Massague, 2015).

Cancer cells can acquire pro-metastatic traits through favourable genetic and epigenetic changes, and the resulting distinct cancer cell clones can generate metastatic lesions (Greaves and Maley, 2012; Vanharanta and Massagué, 2013). At a cellular level, cytoskeletal alterations, matrix metalloproteinases (MMPs) and cathepsins have been implicated in the early stages of metastatic colonisation. Different niches have been described that can support infiltrating cancer cells. For example, pre-metastatic niches formed by signals from the primary tumour and its microenvironment may have a role in recruiting stromal cells that facilitate tumour growth. Metastatic growth is supported by activation of stem cell growth and survival pathways as well as evasion of immune defences e.g. cytotoxic T cells and Natural Killer T (NKT) cells (Massagué and Obenauf, 2016; Fares et al., 2020).

Many cancer subtypes metastasise to specific organs, through a process termed "organotropism" or "organ-specific metastasis". For example, in breast cancer, the luminal subtype is associated with bone metastasis. Human epidermal growth factor receptor 2 (HER2)-positive and triple-negative cancers have a higher propensity to metastasise to visceral organs such as the liver and lung (Soni et al., 2015; Wu et al., 2017). Small cell lung cancer is more likely to metastasise to the liver than other histological subtypes (Riihimäki et al., 2014). Organotropism is regulated by tumour-intrinsic properties, organ-specific niches and the interaction between tumour cells and the host microenvironment. Stephen Paget first described the notion of cells with metastatic potential (seed) and a suitable organ environment (soil) as essential components of metastasis, this was based on the observation that different cancers show a predilection for metastasis to different organs (Paget, 1989). In CRC, the mechanisms dictating organ-specific metastasis e.g. haematogenous spread to the liver versus transcoelomic spread to the peritoneum are poorly understood. Mucinous and signet ring CRC is more likely to metastasise to the peritoneum (Riihimaki et al., 2016) and epidemiological data suggest that rectal cancer more commonly metastasises to the lung than colonic cancer (Riihimaki et al., 2016; Mitry et al., 2010; van der Geest et al., 2015). A common hypothesis presented in the literature is that venous drainage of the colon occurs via the portal venous system to the liver whereas the lower rectum drains into the central venous system, where it is circulated directly to the lungs. However, the biological mechanisms that determine the site and order of organ metastasis are unclear. Tumour intrinsic factors that favour liver organotropism have been identified and the genes and pathways implicated in this process have been reviewed by Gao and colleagues (Gao et al., 2019). Organ-specific environments play important roles in the early stages of cancer cell colonisation with the subsequently acquired ability of cancer cells to manipulate and remodel the host organ microenvironment. It is considered that intraperitoneal metastases develop as a result of tumour invasion through the bowel wall and breach of the visceral peritoneum or, iatrogenically, as a result of

intraoperative escape of tumour emboli from dissected lymphovascular structures, or blood spillage from the operative field, causing tumour cell spillage into the peritoneal cavity (Koppe et al., 2006). Neumann and colleagues reported that expression of cancer stem cell markers is associated with exclusive hepatic metastasis and combined hepatic and peritoneal metastasis but not with peritoneal metastasis alone, suggesting peritoneal tumour deposits lack a stem cell phenotype which is needed for dissemination (Neumann et al., 2015). However, more recently, Barriuso and colleagues have observed activation of 'stemness' pathways using transcriptomic analysis of peritoneal tumour deposits (Barriuso et al., 2021).

4. Colorectal cancer and the microbiome

The vast majority of CRC cases are sporadic (80%), with the remaining cases being hereditary. Sporadic CRC arises through the accumulation of sequential molecular changes (Vogelstein et al., 1988). Although a direct causal link between microbial infection and CRC has not been established, mounting pre-clinical and clinical evidence suggest the involvement of microbes in the development and progression of CRC. This is unsurprising given the microbial abundance in the large intestine. It has been proposed that some intestinal bacteria act as 'bacterial drivers' with pro-carcinogenic features whilst some behave as passengers or are secondary to the cancer (Tjalsma et al., 2012). Bacterial enzymes have been implicated in the activation and production of pro-carcinogenic metabolites from dietary substrates and the cumulative effect of these metabolites may be implicated in the aetiology of CRC (McBain and Macfarlane, 1998; Louis et al., 2014; Zhang et al., 2021; Kim and Jin, 2001). Major microbial metabolites formed from dietary and environmental compounds have been associated with colorectal cancer initiation/progression. For example, some short-chain fatty acids (SCFA), major bacterial fermentation products, are thought to mitigate colorectal carcinogenesis through anti-inflammatory and apoptotic effects whereas the byproducts of protein fermentation e.g. *N*-nitroso compounds (NOCs), ammonia and polyamines may promote colorectal carcinogenesis through the production of reactive oxygen species, inflammation and direct genotoxicity (Louis et al., 2014). There has been a rapid expansion in evidence linking the intestinal microbiome to colorectal neoplasia from benign adenoma through to invasive carcinoma. Evidence from metagenomic analyses suggests CRC is associated with a state of pathological microbial symbiosis (Feng et al., 2015; Nakatsu et al., 2015; Liang et al., 2017; Yu et al., 2017a). Improved understanding of the role of the intestinal microbiome in patients with CRC has potential clinical implications. For example, the impact of postoperative therapeutic/dietary modification of the microbiome is unknown and may influence the risk of developing recurrent disease, metachronous cancer and overall patient well-being.

The intestinal microbiota is associated with CRC development, progression and response to treatment, with changes in the abundance of specific bacteria in patients with CRC being reported (Tilg et al., 2018; Wong and Yu, 2019). *Fusobacterium nucleatum* is a Gram-negative rod-shaped obligate anaerobe considered to be a pro-oncogenic 'driver' and is reportedly one of the most prevalent bacterial strains in CRC. Its association with tumourigenesis is supported by both pre-clinical and clinical studies (Tilg et al., 2018). Preclinical studies have also proposed an association between the carcinogenic role of other organisms such as enterotoxigenic *Bacteroides fragilis* and *Escherichia coli* and the development of CRC (Tilg et al., 2018; Garrett, 2019). A comprehensive list of the most enriched bacterial genera and species associated with colorectal neoplasia has been reviewed by Ternes and colleagues (Ternes et al., 2020). Mechanisms by which bacteria may promote tumourigenesis include (i) direct interaction with host cells resulting in attachment and invasion, ii) bacterial metabolism and secreted metabolic products and (iii) modulation of the anti-tumour immune response (Louis et al., 2014; Ternes et al., 2020).

Several studies have demonstrated the enrichment of *F. nucleatum* in stool samples from patients with colorectal neoplasia as well as human colorectal adenoma and carcinoma tissue relative to adjacent normal tissue (Kostic et al., 2012; Castellarin et al., 2012; Kostic et al., 2013; Mima et al., 2016a); the presence of *F. nucleatum* in CRC has been associated with proximal tumour location and poor prognosis (Mima et al., 2016a; Tahara et al., 2014) as well as increased tumour growth in vivo (Kostic et al., 2013; Bullman et al., 2018). For example, accelerated tumour growth was observed in *F. nucleatum*-treated nude mice xenografts; $Apc^{min/+}$ mice gavaged with *F. nucleatum* developed significantly more colorectal tumours and reduced survival compared to controls (Yang et al., 2014). Several mechanisms relating to *Fusobacterium* tumourigenesis in CRC have been proposed. For example, HCT116 CRC cells infected with *F. nucleatum* increased expression of miR21 by activating TLR4 signalling to MYD88 with subsequent activation of canonical NF-κB transcription factors (Yang et al., 2014). Other studies have also reported increased NF-κB activation in human CRC samples with high abundance of *Fusobacterium* compared to tumours with low expression (Kostic et al., 2013). One possible oncogenic mechanism of *Fusobacterium* involving the adhesin FadA was investigated by Rubinstein and colleagues where they observed FadA binds to E-cadherin on CRC cells, facilitating attachment of *Fusobacterium* and infection of the cell with subsequent activation of oncogenic Wnt/ß-catenin signalling (Rubinstein et al., 2013). They go on to propose a "two-hit model" in CRC development. The accumulation of driver mutations as described in the adenoma-carcinoma model as the first 'hit', and *F. nucleatum* as the second 'hit' which becomes a facilitator of cancer progression (Rubinstein et al., 2019). *F. nucleatum* may also enhance the ability of a tumour to evade the immune system; tumours with *F. nucleatum* inhibited NK cell toxicity and tumour infiltrating lymphocytes via interaction of the *F. nucleatum* protein Fap2 with inhibitory immune receptor TIGIT (Gur et al., 2015). Altogether, there is substantial evidence implicating *F. nucleatum* as an oncogenic "driver" in the development of CRC.

5. Bacteria and colorectal metastasis

5.1. Potential mechanisms

There is increasing recognition that microbes are an intrinsic component of the tumour microenvironment. However, the mechanisms by which microbes may contribute to the development of distant colorectal metastasis are unclear, much of which can be attributed to the technical difficulties of investigating microbiota and the environments they thrive in. The liver is intimately linked with the gastrointestinal tract via the portal venous circulation and is essential for processes related to digestion such as nutrient metabolism and clearance of bacterial metabolites. *In vitro* studies where CRC cells co-cultivated with specific bacterial strains from CRC biopsies show changes in the expression of genes associated with epithelial-mesenchymal transition (EMT) (Wachsmannsmanova, 2019), suggesting bacteria may be capable of supporting EMT and cancer progression.

A link between intestinal bacteria-controlled bile acid metabolism and liver antitumor immunosurveillance has been observed in a non-colorectal model of liver metastases in which altering the intestinal bacteria with antibiotics (specifically, eradication of gram-positive bacteria which mediates primary to secondary bile acid conversion), induced a liver-selective anti-tumour effect with the selective increase of hepatic C-X-C Motif Chemokine Receptor 6 ($CXCR6^{+}$) NKT cells. This provides a link between intestinal microbes, their metabolites and immune cell infiltrates which would be of interest to study in the context of CRC liver metastasis (Chi Ma et al., 2018). In a separate study, antibiotic-treated mice undergoing mesenteric implantation of MC38 cells followed by intragastric *E.coli* administration, developed a greater number and size of liver metastasis. This was associated with high lipopolysaccharide (LPS) secretion and overexpression of metastasis-related secretory protein cathepsin K (CTSK). CTSK accelerated M2 polarisation of tumour-associated macrophages (TAMs) in a

TLR4-mTOR-dependent pathway. This study highlights the role intestinal dysbiosis may have on the initiation and progression of CRC through a gut-liver axis (Li et al., 2019).

Anti-tumour properties of beneficial microbes have also been reported. For example, in vitro studies have observed lactic acid bacteria such as *Lactobacillus* and their metabolites can suppress the growth of CRC cells through anti-proliferative and apoptotic properties (Tiptiri-i-Kourpeti et al., 2016; Chondrou et al., 2018; Ghanavati et al., 2020). HT-29 cells treated with a *Lactobacilli* 'cocktail' resulted in an anti-proliferative effect by downregulation of genes regulating Notch and Wnt/β-catenin pathways (Ghanavati et al., 2020). *In vitro* studies have observed metabolites from *Lactobacillus planatarum* inhibit CRC cell invasion and migration by downregulating VEGF/MMP signalling pathways. Furthermore, GABA-producing *Lactobacillus planatarum* has anti-proliferative and apoptotic activity in 5-FU resistant HT-29 cells via $GABA_B$ receptor signalling (An et al., 2021).

5.2. Lipopolysaccharide and colorectal metastasis

Lipopolysaccharide (LPS) is the main component of the outer membrane of Gram-negative microbiota commonly found in the human gut. Raised circulating LPS and markers of inflammation have been observed in patients with colorectal adenoma and carcinoma (Kang et al., 2013; de Waal et al., 2020). Several studies have highlighted the role of LPS in promoting tumourigenesis. LPS concentration is higher in CRC compared to normal adjacent tissue and patients with lymph node metastasis had high concentrations of LPS compared to those with no lymph node metastasis. *In vitro*, LPS promotes tumour cell extracellular matrix adhesion and invasion through activation of the urokinase plasminogen activator (u-PA) system, a key component of extracellular matrix breakdown, in a TLR4/NF-κB-dependent manner (Killeen et al., 2009). Further studies have observed that LPS stimulation increases migration and invasion of SW480 and HCT116 cells but did not affect cell proliferation. VEGF-C is a key regulator of lymphangiogenesis and expression of VEGF-C is associated with lymph node metastasis in CRC tissue (Akagi et al., 2000; Li et al., 2011). Zhu and colleagues observed that LPS has a role in cell migration, invasion and lymphangiogenesis through increased VEGF-C secretion via TLR4-NF-κB/JNK signalling (Zhu et al., 2016). *In vivo*, mice injected with LPS-treated HT-29 cells had significantly more liver metastasis compared to those with untreated HT-29 cells. In this study, stimulation of TLR4/MD2 complex by LPS activated PI3K/AKT signalling and downstream β1 integrin activity, resulting in increased adhesiveness and metastatic capacity of CRC cells (Hsu et al., 2011). Song and colleagues observed high LPS in orthoptic CRC tissue is associated with reduced response to anti-PD-L1 monoclonal antibody (mAb) treatment, however, clearance of intestinal Gram-negative bacteria or inhibition of TLR4 resulted in boosted T-cell infiltration. Targeting of tumour LPS with an LPS-targeting fusion protein significantly reversed an immunosuppressive tumour microenvironment, improved anti-PD-L1 mAb treatment efficacy, prevented CRC liver metastasis and attenuated any metastatic growth in the liver, highlighting the role of LPS in the metastasis of CRC via the gut-liver axis (Song et al., 2018).

5.3. Fusobacterium and colorectal metastasis

Studies have demonstrated that *Fusobacterium* is associated with distant metastases from primary CRC. For example, Bullman and colleagues observed microbiome stability between primary colorectal tumours and matched liver metastasis. Using RNA sequencing, they observed the same *Fusobacterium* species in primary tumour and matched liver metastasis as well as other Gram-negative anaerobic organisms such as *Bacteroides fragilis*, *Bacteroides thetaiotaomicron* and oral anaerobes *Prevotella intermedia* and *Selenomonas sputigena.* In this study, patient-derived samples infected with *Fusobacterium* were more likely to result in successfully established xenografts compared to *Fusobacterium* negative samples. However, *Fusobacterium* load was not predictive for cancer recurrence or disease stability in 77 patients with primary CRC (Bullman et al., 2018). Bullman and colleagues hypothesised that *Fusobacterium* travels with primary tumour cells to distant sites. This finding is supported by Chen and colleagues who observed a greater abundance of *F. nucleatum* in matched lymph nodes with metastasis compared to lymph nodes without metastasis (Chen et al., 2020a). Similar to primary CRC, *F. nucleatum* positivity in resected colorectal liver metastasis tissue is associated with reduced T cell density ($CD8^+$) as well as increased density of myeloid-derived suppressor cells (Sakamoto et al., 2021). Once again this highlights the immunosuppressive role *F. nucleatum* may have on the tumour microenvironment.

Distinct mechanisms for how *F. nucleatum* may promote the formation of metastases have been proposed. These include induction of interleukin-8 (IL-8) and C-X-C Motif Chemokine Ligand 1 (CXCL1), activation of autophagy through upregulation of caspase activation and recruitment domain 3 (CARD3) and activation of NF-κB-dependent Keratin7-antisense (KRT7-AS) (Chen et al., 2020a; Casasanta et al., 2020; Chen et al., 2020b). More recently, a novel mechanism has been proposed by Guo and colleagues who observed *F. nucleatum* infection increased exosome secretion, enhanced migration of HCT116/SW480 CRC cells and stimulated miR-1246/92b-3p/27a-3p-enriched and CXCL16/RhoA/IL-8-enriched exosomes in vitro. These findings were supported by two in vivo models injected with supernatant of *F. nucleatum* infected HCT116 cells which resulted in systemic metastasis when compared to controls. In patients, circulating exosomal miR-1246/92b-3p/27a-3p and CXCL16 were closely associated with *Fusobacterium* abundance and CRC tumour stage (Guo et al., 2021). Human colorectal tumour organoids co-cultured with *F. nucleatum* induced expression of genes associated with cancer metastasis (Kasper et al., 2020). Altogether, these studies demonstrate some of the mechanistic insights into how *F. nucleatum* can promote colorectal metastasis and the pro-tumourigenic effects of microbiota in the tumour microenvironment. Further work is required to understand how these mechanisms could potentially impact chemotherapy efficacy and resistance.

5.4. Gut vascular barrier and colorectal metastasis

Although sites of CRC metastasis such as the liver do not have a known microbiome, they may be exposed to microorganism-associated molecular patterns (MAMPs) and bacterial metabolites through the enterohepatic circulation (Yoshimoto et al., 2013). The gut-vascular barrier (GVB) is the interface between mucus/epithelial cells, immune cells, endothelial cells and accessory cells such as enteric glial cells and pericytes. The GVB plays a critical role as a physical and immunological barrier against the environment of the intestinal lumen and inhabitant microbiota. Disruption of the GVB can lead to systemic dissemination of microbes and microbe-derived metabolites leading to low-grade systemic inflammation (Brescia and Rescigno, 2021). Gut vascular impairment has been associated with several systemic diseases including the development of a "pre-metastatic" niche in CRC liver metastasis. Distant metastases are facilitated by the formation of a "premetastatic niche" that sees complex molecular and cellular changes at distant sites which attract circulating tumour cells and support future metastatic tumour growth (Peinado et al., 2017; Hiratsuka et al., 2006, 2008; Kaplan et al., 2010; Hoshino et al., 2015).

Bertocchi and colleagues proposed a "pre-metastatic niche" in the liver is induced by bacterial dissemination from the primary colorectal tumour and this may be attributed to gut-vascular barrier impairment. Specifically, they observed that in patients with CRC, high expression of PV-1 (an endothelial marker of impaired GVB) was associated with a statistically higher bacterial load in liver metastasis detected by fluorescence in situ 16 S rRNA-hybridisation, development of metachronous liver metastasis and reduction in disease-free and progression-free survival (Bertocchi et al., 2021). In an $APC^{Min/+}$C3arKO mouse model of CRC, increased PV-1 detection correlated with bacterial dissemination

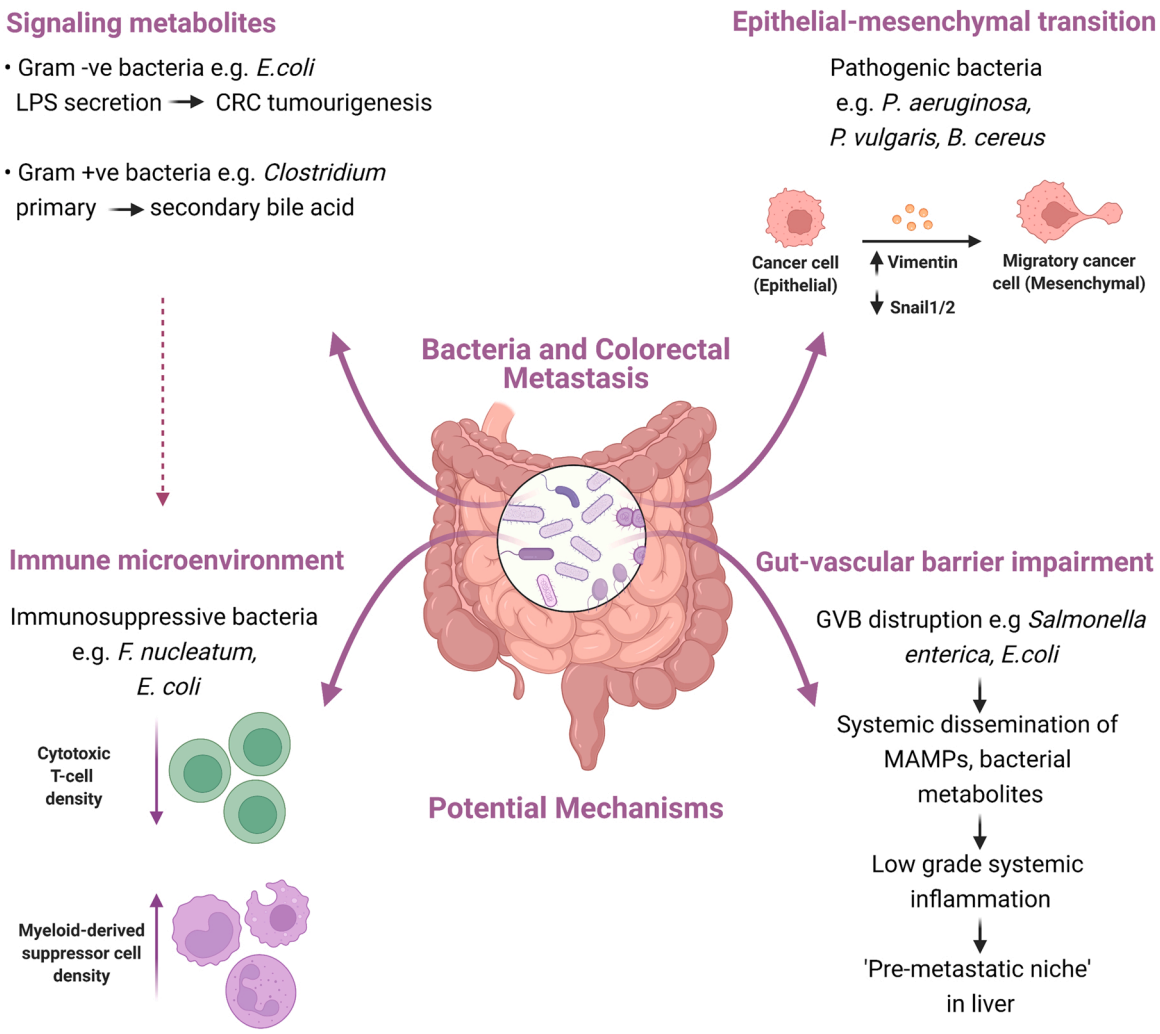

Fig. 2. Bacteria and colorectal cancer metastasis – potential mechanisms. Created with Biorender.com.

to the liver, recruitment of innate immune cells and development of an inflammatory environment consistent with a "premetastatic niche", before the formation of any liver metastases. In addition, it was reported that murine primary CRC and liver metastasis are mainly colonised by *E. coli* C17 which can disrupt the gut-vascular barrier and migrate to the liver. *E. coli* C17 was statistically more abundant in highly expressing PV-1 metastatic tissue compared to patients with low PV-1 expression. It was of particular interest that introduction of the beneficial bacterium *Lactobacillus paracasei* reduced murine PV-1 detection and hepatic innate immune cell infiltration compared to untreated controls (Bertocchi et al., 2021), highlighting the differing roles of microbes, the complexity of how they interact within a microenvironment and how microbiota contribute to the integrity of the GVB. There are several reports providing further evidence that there is a conserved microbiome between primary CRC and corresponding liver metastasis. Therefore, there is a mounting body of evidence to support the hypothesis that targeting the GVB or specific bacterial populations to halt the development of a "pre-metastatic niche" in high-risk patients with CRC may prevent the development of liver metastasis. In addition, future studies need to consider the role of PV-1 as a predictive marker for the development of liver metastasis.

Dysbiosis of the intestinal microbiome has been associated with impairment of the GVB. Szentkuti and colleagues observed a strong influence of the intestinal microbiota on mucin content, thickness, composition and structure in the pre-epithelial colonic mucus layer (Szentkuti et al., 1990). This barrier may be disrupted in patients undergoing treatment for CRC. Significant physiological insult to the gut may result from drug cytotoxicity, antibiotic treatment, surgical resection of the bowel and post-operative complciations. In addition, there may be associated reduction in dietary intake which may contribute further to microbial dysbiosis. The studies outlined above implicate the intestinal microbiome as a source of disease and a contributor to the development of CRC metastasis but also as a potentially protective mechanism that can be exploited. Studies may wish to understand the benefit of 'gut microbiome screening' before commencing any treatments with the aim of understanding if microbiome-targeted interventions, such as dietary modifications and supplements with pro/pre-biotics can improve GVB function and survival from CRC. For example, in mice with colorectal liver metastasis, supplementation with SCFA (a beneficial product of fibre fermentation by intestinal microbiota), increased the relative abundance of probiotic SCFA-producing bacteria, improved host anti-tumour responses and reduced the number of colorectal liver metastasis (Ma et al., 2020). Potential mechanisms by which bacteria may promote colorectal cancer metastasis are shown in Fig. 2.

Whilst potential microbial mechanisms that facilitate metastasis of CRC to the liver are being uncovered, the molecular steps which underpin the development of colorectal peritoneal metastasis remain unclear. Interestingly, patients with ovarian cancer have a unique peritoneal fluid microbial profile compared to those with a benign mass (Miao et al., 2020). Primary colorectal tumour perforation arises as a result of cancer invasion through the bowel wall and breach of the

visceral peritoneum. It is plausible that bacterial contamination of the peritoneal cavity with colonic organisms has a role in creating a favourable environment that enables spilled primary tumour cells to attach and invade the mesothelial lining of the peritoneal cavity. Microbiota that commonly causes peritoneal infection due to colonic perforation have also been implicated in colorectal tumourigenesis (Ternes et al., 2020). The mechanisms that govern the attachment of CRC cells to mesothelial cells and penetration into submesothelial stroma are virtually unknown. This is in contrast to peritoneal ovarian carcinomatosis where cell adhesion molecules (CAMs) (Slack-Davis et al., 2009) and integrins that mediate cell-cell and cell-extracellular matrix (ECM) interactions are well studied. Several integrins have been identified as important facilitators in the metastasis of ovarian cancer to the mesothelial cells of the peritoneum (Shibata et al., 1997). The ECM is a key dynamic component of structural and biochemical tumour support (Winkler et al., 2020). Bacterial proteases can behave in an intra- and extracellular role and can contribute to the organism's virulence. Bacterial proteases can cause ECM degradation and remodelling as a result of tissue inflammation, disruption of host physical barriers and impairment of innate and acquired host immune responses (Lyczak et al., 2000). Bacterial endotoxin, LPS, is found on the surface of most Gram-negative bacteria. *In vitro* experiments using CRC cell lines would suggest it has a role in promoting cell adhesion and ECM invasion through TLR-4 and NF-κB-dependent activation of the u-PA system, which has key roles in ECM remodelling, tumour progression and metastasis (Killeen et al., 2009; Wang et al., 2003). Exploring the interactions between microbiota, CRC cells, mesothelial cells and ECM may be important in overcoming the limitations in current treatments available for patients with colorectal or indeed ovarian peritoneal metastases.

6. Microbes and cancer therapies

In metastatic CRC, cytotoxic chemotherapy and targeted molecular therapies are used to inhibit tumour growth and prevent the development of further metastasis to increase patient survival whilst preserving or improving quality of life. Innate and acquired treatment resistance remains one of the key challenges in managing incurable metastatic CRC. A growing body of evidence suggests microbes can influence drug metabolism and the efficacy of cancer treatments, including those agents used in patients with metastatic CRC (Noriho Iida et al., 2013; García-González et al., 2017; Alexander et al., 2017). *In vitro* experiments have shown bacterial metabolites have the potential to modulate responses to treatment (An et al., 2021). Faecal samples from patients with CRC have higher levels of the bacterial enzyme β-glucuronidase compared to healthy controls (Kim and Jin, 2001). Indeed, the gastrointestinal side effects of widely used Irinotecan are the result of β-glucuronidase causing reactivation of the drug into its active metabolite (SN-38) in the GI tract, leading to gastrointestinal tract insult and dose-limiting side effects (Wallace et al., 2010, 2015). Randomised controlled trials continue to examine the use of probiotics with standard chemotherapy and their impact on the incidence of diarrhoea and response rate (McQuade et al., 2019).

Tumours arising from the left and right colon have distinct clinical, anatomical and molecular characteristics (Benedix et al., 2014, 2011; Patel et al., 2018). A retrospective analysis of six randomised trials demonstrated patients with metastatic, left-sided and wild-type KRAS tumours had a significant benefit from treatment with chemotherapy and anti-EGFR therapy compared with no benefit for those patients with right-sided tumours (Arnold et al., 2017). One possible explanation for this could be due to differences in microbiota composition and organisation between proximal and distal CRC (Dejea et al., 2014; Flemer et al., 2017). Zmora and colleagues observed the microbiome can vary at different anatomical locations of the gastrointestinal tract (and between mucosa, lumen and faeces) (Zmora et al., 2018) and therefore it is plausible that the effects of microbiota in different locations may impact cancer treatments. A high abundance of *F. nucleatum* in CRC is associated with proximal tumour location (Mima et al., 2016b), distinct molecular features (Mima et al., 2016a; Tahara et al., 2014), a lower density of $CD3^+$ T-cells (or 'impaired adaptive immune response') (Mima et al., 2015) and chemoresistance. Yu and colleagues observed *F. nucleatum* was enriched in patients with recurrent CRC compared to those without recurrence and hypothesised that an abundance of *F. nucleatum* may promote CRC chemoresistance. This group observed *F. nucleatum* promoted chemoresistance (5-FU, oxaliplatin) via targeted TLR4 and MYD88 innate immune signalling and genomic loss of microRNAs to activate autophagy pathways (Yu et al., 2017b).

To date, there is little specific experimental evidence to suggest intestinal microbiota can alter the response to EGFR-targeted therapy. Angiogenesis-mediated function for intestinal microbiota has been reported (Schirbel et al., 2013; Suh et al., 2019) and preclinical studies suggest probiotics can modulate angiogenesis by regulating VEGFR signalling, although the role of probiotics in patients receiving anti-VEGFR therapy for metastatic CRC is unclear. A small retrospective cohort study has suggested antibiotic exposure could be associated with reduced mortality in patients with metastatic CRC treated with bevacizumab (Lu et al., 2019), this observation requires further validation.

In a study by Geller and colleagues, several bacterial species conferred gemcitabine resistance when cultured with RKO human CRC cells. The Gammaproteobacteria class have the potential to confer CDD_L-mediated gemcitabine resistance. This was demonstrated in a colon cancer mouse model injected with *E.coli* strain Nissle 1917 whereby subsequent treatment with gemcitabine and antibiotic (ciprofloxacin), showed the absence of detectable bacteria and marked anti-tumour response when compared to those mice treated with gemcitabine alone. The authors also observed expression of bacteria is a component of the pancreatic ductal adenocarcinoma tumour microenvironment and when cultured from fresh tissue, could render HCT116 human colon cancer cell lines fully resistant to gemcitabine. Although these results do not directly involve the investigation of colorectal metastasis, it does highlight the importance of understanding the bacteria at play within a tumour microenvironment and how they may interfere with drug metabolism and efficacy (Geller et al., 2018).

There is compelling evidence that host immunity and intestinal microbiome symbiosis have a key role in determining innate and adaptive immune responses at a local and systemic level, with microbiota shaping the immune system as a whole (Malin et al., 2015; Honda and Littman, 2016; SVancheswaran Gopalakrishnan1 et al., 2021; Zheng et al., 2020). Microsatellite instability (MSI) is found in 4–5% of patients with metastatic CRC and is an important predictive biomarker for response to immunotherapy (Dekker et al., 2019; Overman et al., 2017, 2018). Increasing pre-clinical and clinical data support the role of the intestinal microbiome in modulating immune responses and efficacy of immunotherapy, with specific bacteria correlated to the immunotherapeutic response. The detrimental effect of antibiotic consumption when receiving immunotherapy has been highlighted in some malignancies (Derosa et al., 2018). Pre-clinical and clinical studies suggest the composition of the intestinal microbiome can influence response to anti-programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) therapy (Vétizou et al., 2016; Gopalakrishnan and Spencer LN, 2018; Matson et al., 2018). As such, promising results have been observed during a phase one clinical trial investigating the safety and feasibility of faecal microbiota transplantation (FMT) and reinduction of anti-PD1 immunotherapy in patients with refractory metastatic melanoma (Baruch et al., 2021). Evidence for the relevance of this to metastatic CRC is awaited.

Microbiome-personalised data is fast becoming an area of research moving into the precision oncology setting and whilst it is not the aim of this review to discuss how to optimally assess the intestinal microbiome (faecal v mucosal, 16 S rRNA sequencing v metagenomic shotgun sequencing) or how to optimally modulate intestinal microbiota (diet/prebiotics/probiotics), these are important considerations for future

Table 1
Intestinal microbiome modulation clinical trials in Colorectal Cancer. RCT (Randomised controlled trial), OS (overall survival), PUFA (polyunsaturated fatty acids), QoL (Quality of Life), PFS (Progression free survival), FMT (Faecal microbiota transplant).

	Condition	Patients	Intervention	Outcome	Status	Results (Location)
Preoperative						
NCT04013841	Patients with colorectal cancer in the preoperative period	60	RCT: Oral agents for mechanical bowel preparation prior to left-sided colorectal resection vs enema	Intestinal microbiome composition	Recruiting	- (Lithuania)
NCT01895530	Patients with colorectal cancer in the preoperative period	33	RCT: Probiotic *(Saccharomyces boulardii*) vs no probiotic	Primary: Change in cytokine gene expression Secondary: Postoperative complications	Completed	- (Brazil)
NCT01609660	Patients with colorectal cancer in the preoperative period	33	RCT: Probiotic *(Saccharomyces boulardii)* vs no probiotic	Mucosal cytokine	Completed	- (Brazil)
NCT04281667	Patients with colorectal cancer in the preoperative period	604	RCT: Mechanical bowel preparation and oral antibiotics vs mechanical bowel preparation only	Primary: Comprehensive Complication Index Secondary: Surgical Site Infection; OS	Recruiting	- (Finland)
NCT01916239	Patients with colorectal cancer in the preoperative period	60	RCT: Pomegranate extract formulations pre-surgery	Primary: Phenolics and derived metabolites; Gene expression profiling Secondary: Circulating IGF-1 and CEA levels; Safety; microRNA expression profiling	Completed	- (Spain)
Perioperative						
NCT00936572	Patients with colorectal cancer in the perioperative period	35	RCT: High/low dose probiotics vs placebo	Primary: Colonic microflora; Gastrointestinal function Secondary: Immune and inflammatory response; Bacterial translocation	Completed	- (Italy)
Postoperative						
NCT03782428	Patients with colorectal cancer in the postoperative period	52	RCT: Probiotics vs placebo	Primary: Level of circulating inflammatory cytokines Secondary: Incidence of diarrhoea	Completed	- (Malaysia)
NCT04821258	Patients with colorectal cancer in the postoperative period	144	RCT: MICODIGEST 2.0 vs placebo	Primary: Complication rate Secondary: Adverse effects; faecal microbiome	Not yet recruiting	- (Spain)
NCT04869956	Patients with colorectal cancer in the postoperative period	50	RCT: High-fibre diet rich in PUFAs vs standard nutritional recommendations	Primary: Anastomotic leakage, Surgical site infection Secondary: Gut microbiome; Serum inflammation markers	Not yet recruiting	- (Spain)
NCT05039060	Patients with colorectal cancer in the postoperative period	40	RCT: Modified microbiota-accessible carbohydrates diet	Primary: Gut microbiome Secondary: Stool formation pattern; QoL; Gut microbiota metabolite	Not yet recruiting	- (South Korea)
NCT01479907	Patients with colorectal cancer in the postoperative period	100	RCT: Synbiotics (multistrain plus fibre) vs placebo	QoL	Completed	- (Greece)
Chemotherapy						
NCT02169388	Patients with colorectal cancer treated with chemotherapy	30	RCT: Probiotics (clostridium butyricum) vs placebo	Primary: Composition of stool microorganisms; Adverse effects Secondary: Changes in immune status indexes	Unknown status	- (China)
NCT00197873	Patients with colorectal cancer treated with chemotherapy	84	RCT: Probiotic *(Lactobacillus Rhamnosus)* vs placebo	Primary: Incidence of diarrhoea Secondary: Toxicity; Response rate to supplementation; Resectability of liver metastases	Completed	- (Finland)
NCT01410955	Patients with colorectal cancer treated with chemotherapy	46	RCT: Probiotic (Dophilus™) vs placebo	Incidence of diarrhoea	Completed	- (Slovakia)
NCT04264676	Patients with colorectal cancer treated with postoperative chemotherapy	294	RCT: Metronidazole vs placebo	Primary: Disease free survival Secondary: OS; Recurrence rate	Recruiting	- (China)
NCT02706184	Patients with gastric/ colorectal cancer treated with chemotherapy	20	RCT: E. coli Nissle suspension vs placebo	Primary: Toxicity criteria for diarrhoea Secondary: QoL; Stool microbiome	Completed	- (Germany)
NCT04021589	Patients with metastatic colorectal cancer treated with chemotherapy	50	RCT: Probiotic (Weileshu) vs usual care (chemotherapy)	Primary: PFS Secondary: OS	Recruiting	- (China)
NCT03705442	Patients with metastatic colorectal cancer treated with chemotherapy	76	RCT: Omni-Biotic 10 vs placebo	Primary: Incidence of grade III/IV diarrhoea Secondary: Zonulin/ vitamin D concentration; QoL	Unknown status	- (Croatia)
NCT04131803	Patients with metastatic colorectal cancer treated with chemotherapy	140	RCT: Probiotic (bifidobacterium trifidum) and standard therapy vs standard therapy	Objective response rate	Not yet recruiting	- (China)
NCT01477866	Patients with colorectal cancer treated with surgery and/or chemotherapy	250	RCT: CITOGENEX *(Lactobacillus* Casei and *Bifidobacterium lactis)* vs placebo	Mortality	Suspended	- (Italy)
NCT04729322	Patients with metastatic colorectal cancer treated	15	Non-RCT: FMT and pembrolizumab vs FMT and nivolumab	Primary: Immune-Modified Response Evaluation Criteria in Solid Tumours (iRECIST)	Recruiting	- (USA)

(*continued on next page*)

Table 1 (*continued*)

	Condition	Patients	Intervention	Outcome	Status	Results (Location)
	with surgery and/or chemotherapy					
NCT03785210	Patients with metastatic colorectal cancer treated with surgery and/or chemotherapy	27	Single-arm: Nivolumab, tadalafil and oral vancomycin	Primary: Overall Response Secondary: Safety; OS	Recruiting	- (USA)
Radiation						
NCT03742596	Patients with colorectal cancer treated with radiotherapy	40	Non-RCT: Probiotic formula capsule vs placebo	Primary: Level of serum immunoglobulins/ cytokines Secondary: QoL; Gastrointestinal toxicity	Recruiting	- (Jordan)

studies. Modulation of the microbiome to improve therapeutic response in cancer has been carefully reviewed by McQuade and colleagues (McQuade et al., 2019). Several studies have investigated the role and impact of probiotics on the intestinal microbiome. Importantly, Zmora and colleagues have demonstrated that in people who consumed a specific 11-strain probiotic formulation, probiotic mucosal colonisation was predicted by host and indigenous intestinal microbial features (Zmora et al., 2018). This study emphasises the complexity of manipulating the intestinal microbiome and how the "one size fits all" approach will simply not work. Several clinical trials are investigating how modulation of intestinal microbiota in patients undergoing treatment for CRC may impact a variety of clinical outcomes (Table 1). There is mechanistic evidence which highlights the intestinal and tumour microbiome is important in defining the efficacy and toxicity of chemotherapeutic agents, this will need to be factored in when designing future clinical trials (Alexander et al., 2017). Clinical trials may wish to incorporate an assessment of the intestinal microbiome on a routine basis, this will expand our understanding of the potential impact of specific treatments and combinations thereof.

7. The tumour microbiome

Most microbiome studies in humans are in reference to the intestinal (faecal) microbiome where faecal samples are considered a proxy to the microbial phenotype of the patient's intestine. When Flemer and colleagues compared the mucosal microbiota in CRC with paired healthy tissue, they observed similarities with regard to individual taxa and overall composition, suggesting microbial changes are not limited to the tumour microenvironment or caused by the tumour but instead reflect that microbial changes are involved at the early stages of CRC development (Flemer et al., 2017). Although there are limited studies that have examined paired mucosal and faecal samples, there is evidence that suggests the mucosal microbiota at least partially reflects those found in faeces (Flemer et al., 2017; Zeller et al., 2014). In one of the first studies of its kind by Marongiu and colleagues, they observed a differential microbial landscape in colorectal liver metastasis, paired primary CRC and normal tissue using metagenomic analysis (Marongiu et al., 2021). Deciphering key microbiome signatures at different stages of cancer diagnosis may impact prognostication, treatment options and surveillance. For example, in pancreatic ductal adenocarcinoma patients, distinct intratumoural microbial diversity and composition was associated with short- and long-term survival (Riquelme et al., 2019).

Despite the technical challenges of investigating intratumoural microbial signals, the presence of bacteria has been demonstrated in a number of solid tumours (Strausmann, 2020). It is unclear whether they have a direct or indirect effect on tumourigenesis or indeed whether they act as innocent bystanders (Cummins and Tangney, 2013). The latter seems less plausible because of the evidence presented in this communication.

8. Conclusions and future direction

The microbiota is a key component of the tumour microenvironment and their role in the development of CRC metastasis is emerging. The role of the gut vascular barrier is an interesting area of research and further work in this field may improve knowledge and support the application of microbiome-targeted interventions. Investigation of metagenomic tumour signatures may offer an opportunity to understand patient outcomes, disease relapse and responses to treatment. The study of host-microbe interactions has expanded with the meta-omic approach (Ternes et al., 2020) however insights into the mechanistic pathways by which bacteria influence CRC metastasis will be required to identify treatments that target this process. Further work is required to understand how microbiome-targeted interventions or 'gut prehabilitation' may be used to improve outcomes and side effects as a result of surgery and/or chemotherapy.

There is an increasing body of evidence demonstrating that investigation of the microbiome may have an impact on the treatment of colorectal cancer liver metastasis. It is now clear that the host organ and its particular mini-ecosystem has implications in developing different responses to systemic treatments, for example, to immunotherapies (Yu et al., 2021). The peritoneum, however, remains a poorly understood metastatic site and a better description of the distinct components of the microbiome in peritoneal metastasis may provide mechanistic insights as well as the potential to better define patient prognosis. We envisage that treatments to modify the specific microbiome of pertioneal metastasis may be used to improve the outcomes of treatments based on immunotherapies.

CRediT authorship contribution statement

Meera Patel: Conceptualisation, Writing – original draft, Writing – review & editing. **Milly McAllister**: Writing – original draft, Writing – review & editing. **Raghavendar Nagaraju**: Writing – review & editing. **Sara Samir Foad Al Badran**: Writing – review & editing. **Joanne Edwards**: Writing – review & editing. **Andrew J. McBain**: Conceptualisation, Writing – review & editing. **Jorge Barriuso**: Conceptualisation, Writing – review & editing. **Omer Aziz**: Writing – review & editing.

Conflict of interest statement

All authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.critrevonc.2022.103856.

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