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Review

Microbiome and cancer

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SUMMARY

The human microbiome constitutes a complex multikingdom community that symbiotically interacts with the host across multiple body sites. Host-microbiome interactions impact multiple physiological processes and a variety of multifactorial disease conditions. In the past decade, microbiome communities have been suggested to influence the development, progression, metastasis formation, and treatment response of multiple cancer types. While causal evidence of microbial impacts on cancer biology is only beginning to be unraveled, enhanced molecular understanding of such cancer-modulating interactions and impacts on cancer treatment are considered of major scientific importance and clinical relevance. In this review, we describe the molecular pathogenic mechanisms shared throughout microbial niches that contribute to the initiation and progression of cancer. We highlight advances, limitations, challenges, and prospects in understanding how the microbiome may causally impact cancer and its treatment responsiveness, and how microorganisms or their secreted bioactive metabolites may be potentially harnessed and targeted as precision cancer therapeutics.

MICROBIOME NICHES AND CANCER

The human body harbors an estimated three trillion bacterial members that orchestrate a comprehensive interplay of physiological processes and disease susceptibilities (Sender et al., 2016). Although there is a similar number of bacterial cells as compared with human cells in the body, the 100-fold higher genetic diversity of bacteria encodes for outstanding mechanistic and metabolic competences that influence not only their own microbial niche, but host tissue–specific and immune cell functions (Gilbert et al., 2018). Beyond bacteria, the human microbiome is also composed of eukaryotic fungi and protozoa, and viruses (Ley et al., 2008). Overall, under healthy conditions, the host and its microbiome exist in symbiosis as a metaorganism by providing a nutrient-rich microenvironment in return for aid in digestion and metabolism (Schwabe and Jobin, 2013; Xavier et al., 2020). The gut, skin, and oral microbiomes are known to feature a highly enriched and diverse population of microbes; whereas, the vaginal microbiome is similarly well-studied but is rather characterized by lower diversity with highly specific and dominant microbial members (Cho and Blaser, 2012). Furthermore, organs and tissues, such as the lung, prostate, bladder, breast, liver, and pancreas that were previously considered to be sterile, are now, with the advent of next generation sequencing (NGS), identified as potentially harboring low-biomass microbial populations (Geller et al., 2017; Nejman et al., 2020; Parhi et al., 2020; Poore et al., 2020; Pushalkar et al., 2018). However, the exact nature of whether these microbiomes are natively established from commensal site-specific populations, or represent a transient migration from adjacent sites is still under debate.

The general development and progression of cancer is considered to feature multiple distinguishable yet complementary and often overlapping hallmarks. By sustaining proliferation, evading cell growth suppression, activating invasion and metastatic pathways, enabling replicative immunity, inducing angiogenesis, and resisting autophagy, cancer cells effectively proliferate and evade the immune system (Hanahan and Weinberg, 2000, 2011). While these processes have been extensively studied for decades, potential microbiome impacts on cancer development, progression, and treatment responsiveness remained elusive until recently. In a disease scenario, each microbial niche (outlined in Figure 1) may influence cancer promotion via community-level interactions mediated by altered microbiome configurations (also termed "dysbiosis") (Xuan et al., 2014) (Sobhani et al., 2011) (Farrell et al., 2012), direct interaction of individual members (Koshiol et al., 2016) (Pereira-Marques et al., 2019), or via secreted or modulated metabolites (Ma et al., 2018; Mager et al., 2020; Meisel et al., 2018). Niche-specific microbiome impacts on cancer (Helmink et al., 2019) can be exemplified by oral microbiome modulation of cancers in the oral cavity (oral squamous cell carcinomas [OSCC]), colon (colorectal cancer [CRC]) and pancreas (pancreatic ductal adenocarcinoma [PDAC]). Likewise, the microbiomes of both male and female pelvic organs feature important implications for urological (Altmae et al., 2019) and gynecological (Laniewski et al., 2020) cancers, including prostate (Shrestha et al., 2018), cervical, endometrial, ovarian (Curty et al., 2019; Nene et al., 2019), and bladder (Bajic et al., 2019)

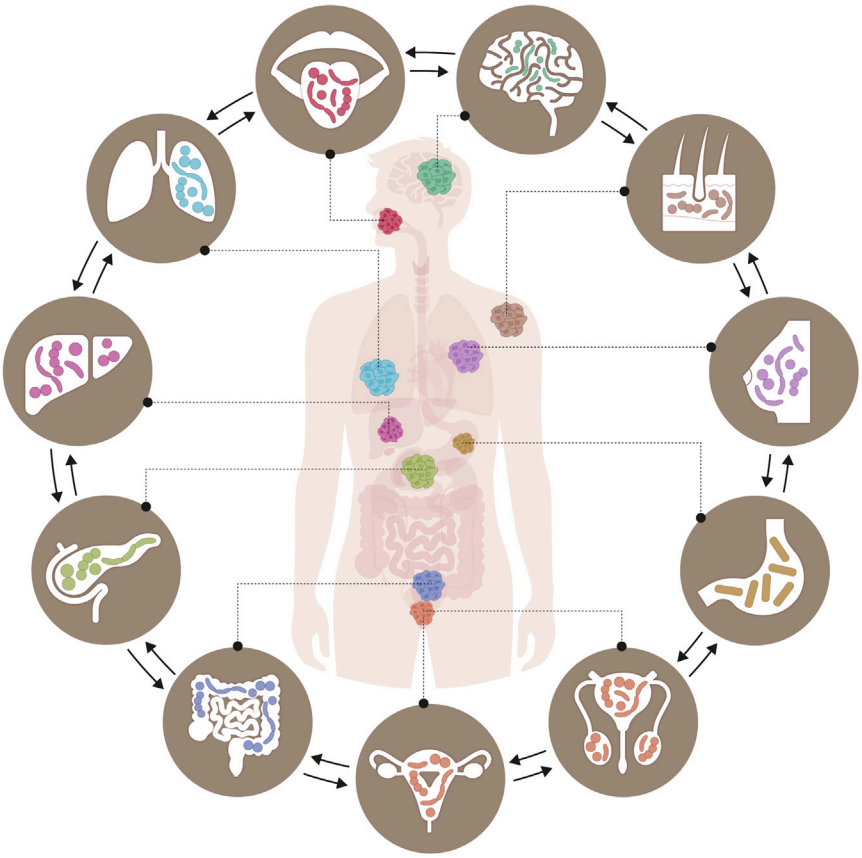

Figure 1. Microbial niches across cancer types

Microbial communities are present in multiple mucosal surfaces and also as low-biomass ecosystems within a variety of cancer types. Microbial members can be shared multidirectionally between niches. For example, Fusobacteria native to the oral cavity are proposed to migrate from the mouth, either through the digestive tract or via the blood stream, to deposit and colonize in the large intestine. Such microbial communities are increasingly suggested to contribute to cancer.

cancers. In addition, the "intratumoral microbiome" may further contribute to local oncogenesis.

INVESTIGATING THE CANCER-ASSOCIATED MICROBIOME

Microbiome composition and function can be characterized by a combination of methodologies, each contributing different facets to the understanding of its complex community structure. In realizing the strengths and limitations of these modalities, one can increasingly integrate their "big data" in defining physiological and pathological associations to human cancers, while minimizing biases and pitfalls.

Next generation sequencing

***16S rRNA* gene sequencing** is a widely used and affordable NGS platform enabling a high-throughput characterization of a large number of technical and biological microbiome replicates for their community structure. However, amplification biases, chimera generation, and methodological variation in amplified regions of the *16S rRNA* gene, data curation, and reference databases, introduce challenges in analytical outputs, data interpretation, and reproducibility across studies (Schloss, 2018). **Shotgun metagenome sequencing** allows not only mapping of the taxonomic composition of a sample, but also the evaluation of genes, including those encoding metabolic pathways, in characterizing the genomic functional potential of commensal microbial communities (Qin et al., 2010). Shotgun metagenome sequencing indiscriminately sequences the entire genome, demanding increased sequencing depth and higher costs, and can provide species- and even strain-level assignment as compared with *16S rRNA* gene sequencing. While compositional reproducibility between studies remains a challenge in shotgun metagenome sequencing, functional outputs of this modality may enable improved concordance between studies (Limeta et al., 2020). Metagenomic sequencing can also be used to computationally reconstruct the composition of the microbial community from the pool of sequence reads and thereby identify new microbial genomes from yet-to-be-named species (Pasolli et al., 2019). Such microbial "dark matter," i.e., members of a microbiome that have not yet been characterized with culture-based methods or with metagenomics, may be of particular relevance for cancer research as tumors represent a highly nonphysiologic environment within a host. **Metatranscriptomics** utilizes NGS to characterize the transcriptional landscape of individual bacteria or the entire microbiome, thereby representing a more direct measure of microbial activity and function, while linking the metagenome and downstream metabolomic and proteomic readouts (Lloyd-Price et al., 2019).

Low-biomass tumor microbiome assessment

Aimed at accurately characterizing the low-biomass intratumoral microbiomes, this NGS modality attempts to account for background noise and contaminations. Multiregion *16S rRNA* gene sequencing on tumor tissues revealed substantial differences in diversity and composition of intratumoral microbiomes between cancer entities (Nejman et al., 2020). This pipeline included stringent tissue processing, DNA extraction, multiple "kitome"-related and amplification controls, and eventually removed 94.3% of bacteria presumed to be contaminants. As another approach, shotgun sequencing data that were generated on tumor tissue biopsies to characterize cancer genetics have been mined to explore a tumor resident microbiome. Analyses of The Cancer Genome Atlas sequencing data revealed microbial signatures in tumors and patients' blood that can be utilized to discriminate cancer subtypes (Poore et al., 2020). However, because of the lack of controls for sample processing and sequencing, a differentiation of endogenous versus

contaminant bacteria can only be achieved by applying bioinformatic decontamination algorithms (Dohlman et al., 2021). Even applying *in silico* decontamination removed large numbers of taxa presumed to represent contaminants (Poore et al., 2020). Given these technical and analytical challenges, it is becoming more common to pursue multiple approaches (Blacher et al., 2019), including *16S rRNA* gene sequencing or multiplex fluorescence *in situ* hybridization, metagenomics, and metabolomics to significantly increase the accuracy and robustness of low-biomass cancer-microbiome assessment.

Nongenomic microbiome characterization

Metabolomics is a mass spectrometry–based method characterizing the thousands of potentially bioactive metabolites generated or modulated by different microbes. Using this method, short-chain fatty acids (SCFAs) like acetate, propionate or butyrate, major metabolites of microbial starch degradation, were observed to stratify progression-free survival in cancer patients treated with anti-PD-1 type immune checkpoint inhibitors (ICIs) (Nomura et al., 2020). **Metaproteomics** characterizes the thousands of peptides and proteins associated with microbiome configurations, but has been so far infrequently applied, and its potential in cancer research still needs to be determined (Neves et al., 2020; Tanca et al., 2017; Zhang et al., 2018). Major histocompatibility complex **peptidomics** enables the characterization of protein-based peptides presented on immune and tumor cells, resulting in modulation of local and systemic immune responses to tumors. This method recently enabled the identification that intratumoral bacterial-related peptides in melanoma tumors are displayed on cancer cell surfaces by human leukocyte antigen (HLA) (Kalaora et al., 2021), while bearing a potential to locally activate an immune response within the tumor microenvironment (TME). In addition to high-throughput sequencing, multicondition microbiome cultivation ("**culturomics**") substantially increases the repertoire of "culturable" commensals, enabling precise taxonomic and functional assessment of individual bacteria in microbial ecosystems. Such culturomic large-scale screening, for instance, revealed that 24% of marketed, nonantibiotic drugs exert growth inhibition of human gut bacteria (Maier et al., 2018).

Cancer-microbiome analysis

Interpreting and analyzing the above multidimensional big datasets and integrating these results into physiological contexts remains a daunting technical and conceptual challenge in cancer-microbiome research. Technically, such big data harmonization and integration is far from trivial, while "in-house" methodologies, even when applied to the same datasets, may lead to nonoverlapping conclusions (Schloss, 2018). Conceptually, only a few putative "driver changes" represent commensals and associated functions that *a priori* contribute to cancer formation, progression, and treatment response, whereas microbial repertoires that are associated with cancer often include "passenger changes" that are secondary to the physiological alterations occurring during cancer. Disentangling causality from correlation, given the diversity in sequencing and data collection methods, represents a formidable challenge to the field. Machine learning and other artificial intelligence–based modalities may enable better recognition of potentially causative signals of clinical relevance (Adlung et al., 2021). By utilizing independent discovery versus validation cohorts, these pipelines may help identify disease or trait-related microbial features while accounting for interindividual variation in microbiome data. Of note, machine learning can identify potentially causal feature contributions within complex big datasets but necessitates experimental validation to reach a sufficient degree of causal certainty. Limitations notwithstanding, we expect an increased use of such artificial intelligence modalities in future cancer-microbiome–focused studies. Importantly, the expected increased availability of longitudinal microbiome data across time in cancer-afflicted individuals throughout their disease and treatment course necessitates that analytical modalities be adapted to tackle the inherent difficulty in assessing microbiome kinetics across time.

CANCER-PROMOTING MICROBES

Currently, only 11 organisms (seven viruses, three platyhelminths, and one bacterium) have been formally recognized as distinct causes of cancer in humans: the Epstein-Barr (EBV), Hepatitis B (HBV), and Hepatitis C (HCV) viruses; Kaposi sarcoma herpesvirus (KSV); human immunodeficiency virus-1 (HIV); human papilloma viruses (HPV); human T cell lymphotropic virus type 1 (HTLV); *Opisthorchis viverrini*; *Clonorchis sinensis*; *Schistosoma haematobium*; and *Helicobacter pylori* (IARC, 2009). Collectively, these microorganisms contribute to cancer progression via a variety of mechanisms, including induction of B cell differentiation, disruption of cell-cycle determination, and immune hyperactivation (in EBV, HBV, HCV, and HIV infection), T cell dysregulation (in EBV and HTLV infection), and direct oncogenesis induced by Hepatitis viruses and KSV in hepatocellular carcinomas and Kaposi sarcoma, respectively. KSV also effectively diminishes apoptosis, as does HPV, through direct involvement of oncogenic proteins. The three carcinogenic flatworm species are strongly associated with cholangiocarcinoma and hepatocellular carcinoma, as well as bladder cancer for *S. haematobium*, through induction of chronic inflammation that leads to oxidative stress and DNA toxicity (IARC, 2009).

Cancer-associated bacteria

While the International Agency for Research on Cancer (IARC)-recognized pro-tumorigenic microbe list has not been updated in more than a decade despite the recent advances in microbiological and microbiome research, recent research suggests that, in addition to *H. pylori,* dozens of microbial species could modulate or contribute to cancer (Sepich-Poore et al., 2021). Notable examples of such microbiome-associated carcinogens are summarized below and depicted in detail in Figure 2.

Helicobacter pylori

H. pylori is an established carcinogen with an age-standardized incidence rate of 8.7 cases per 100,000 individuals per year (de Martel et al., 2020) and is detectable in over half of the world's population (Hooi et al., 2017). Overall, *H. pylori* contributes to peptic ulcers, gastric cancers, and mucosa-associated lymphoid tissue (MALT) lymphomas (Cover and Blaser, 2009; Sugizaki et al., 2018) via its interferences in the Wnt/β-catenin pathways regulating cellular turnover and apoptosis. *H. pylori* can indirectly influence the development of cancer as outlined by the Correa pathway (Correa et al., 1975; Correa and Piazuelo, 2012) via a chronic inflammatory response mediated by the

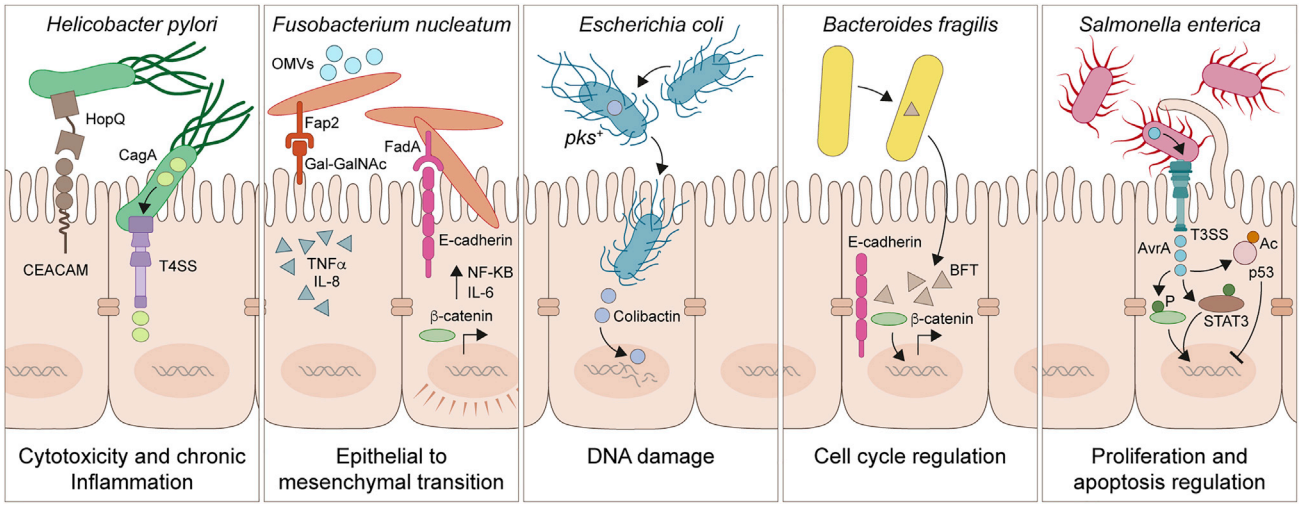

Figure 2. Microbial impacts on neoplastic processes in epithelial cells

Exemplified are several hallmarks of microbial impacts on cancer-related epithelial cell pathways. *H. pylori* binds to gastric epithelial cells by the outer membrane adhesin HopQ binding to CEACAM, whereby virulence factor CagA is directly injected into the epithelial cells via the T4SS. CagA activates Wnt/β-catenin pathways resulting in dysregulated cellular turnover and apoptosis. *F. nucleatum* might trigger cancer via multiple ways. Virulence factors such as the FadA adhesin allow cellular internalization and induction of proinflammatory cascades mediated by NF-κB and IL-6. Fap2, another important adhesin, interacts with D-galactose-β (1–3)-N-acetyl-D-galactosamine (Gal-GalNAc) carbohydrate moieties at the tumor surface to enhance cellular proliferation via Wnt/β-catenin pathway, increase proinflammatory cytokine production, and contribute to EMT, which is a prominent feature of cancer cell invasion, metastasis, stemness, and therapy resistance. Pathogenic *E. coli* has a repertoire of virulence factors and toxins classically associated with pathogenicity, including the secreted genotoxin colibactin. Once internalized by the host cell, it induces interstrand crosslinks and double-strand DNA breaks with pro-tumoral cellular transformations and a mutational signature detected in multiple cancer genomes. Enterotoxigenic *B. fragilis* (ETBF) encodes the BFT, which targets the intestinal cell tight junction, leading to the cleavage of E-cadherin. Consequently, this increases intestinal permeability and induces chronic intestinal inflammation via NF-κB signaling and tissue damage with increase of reactive oxygen species, leading to CRC. *S. enterica* Type 3 secretion system (T3SS) drives an intracellular injection of virulence factors such as the multifunctional effector AvrA that favors tumor formation via activation of cell proliferation triggered by Wnt/β-catenin activation. Further activation of MAPK and AKT pathways is critical to sustain cellular transformation in cancer.

recruitment of polymorphonuclear neutrophils and invasion of mononuclear lymphocytes, stimulating a predominantly Th1-type response by proinflammatory signaling through interleukin (IL)-1B, tumor necrosis factor (TNF)α, and interferon (IFN)γ (Bagheri et al., 2018; Guiney et al., 2003; Hafsi et al., 2004). *H. pylori* attaches to gastric epithelial cells by the outer membrane adhesin HopQ binding to carcinoembryonic antigen-related cell adhesion molecules (CEACAM) (Hamway et al., 2020; Javaheri et al., 2016), whereby CagA is then directly injected into the epithelial cells via the type 4 secretion system (T4SS) (Odenbreit et al., 2000). In the gastric epithelium, it interacts with the oncogenic SHP2 phosphatase and PI3K, thus indicating that *H. pylori* has a direct role in neoplastic transformation of epithelial cells (Hatakeyama, 2017).

Fusobacterium nucleatum

While undertaking an exploratory study to identify bacteria present in colorectal tumors, Castellarin et al. discovered transcripts from *F. nucleatum* at 400 times greater levels in CRC tumor tissues than in matched healthy tissues, indicating that an oral pathogen associated with OSCC might also influence cancer at a distant body site (Castellarin et al., 2012; Kim et al., 2019; Yang et al., 2018). In addition, *F. nucleatum* has been associated with liver metastasis, further broadening its potential reach in cancer (Bullman et al., 2017). *F. nucleatum* binds to host epithelial and endothelial cells via the FadA adhesin (Xu et al., 2007), thereby allowing cellular internalization of the pathogen, induction of proinflammatory cascades mediated by nuclear factor (NF)-κB and IL-6, and a possible route for invasion of OSCC cells (Han et al., 2000, 2004). *In vitro*, outer membrane vesicle (OMV)-producing *F. nucleatum* stimulates inflammation via the secretion of IL-8 and TNFα from colonic epithelial cells (Engevik et al., 2021). Furthermore, *F. nucleatum* localizes at tumor sites and binds via its Fap2 galactose-binding lectin interacting with D-galactose-β(1–3)-N-acetyl-D-galactosamine (Gal-GalNAc) carbohydrate moieties at the tumor surface within CRC (Abed et al., 2016) and breast cancer (Parhi et al., 2020). *F. nucleatum* may also contribute to epithelial-mesenchymal transition (EMT), which is a prominent feature of cancer cell invasion, metastasis, stemness, and therapy resistance (Zhang et al., 2020).

Escherichia coli

E. coli strains contribute to cancer formation by inducing inflammation, oxidative stress, and changes in the cellular niche, coupled with interference and manipulation of the host cell cycle (Bonnet et al., 2014). In addition, colibactin, a genotoxin secreted by pathogenic *E. coli* and encoded by the *pks* pathogenicity island induces interstrand crosslinks and double-strand DNA breaks in eukaryotic epithelial cells (Nougayrede et al., 2006; Buc et al., 2013; Bossuet-Greif et al., 2018; Pleguezuelos-Manzano et al., 2020). Furthermore, *E. coli* may enhance senescence via production of growth factors that promote cellular proliferation and tumor growth, as shown in CRC mouse models (Arthur et al., 2012; Dalmasso et al., 2014) and CRC human biopsies (Dalmasso et al., 2014). Of note, the *E. coli*–induced DNA damage responses, as well as the indirect effects it inflicts on Wnt signaling (both contributing to cellular transformation) are

frequently observed in CRC (Bossuet-Greif et al., 2018; Dalmasso et al., 2014; Dziubanska-Kusibab et al., 2020; Iftekhar et al., 2021).

Bacteroides fragilis

While *B. fragilis* is a symbiont flourishing the entire length of the colon, enterotoxigenic *B. fragilis* (ETBF) strains have been associated with induction of colitis and colon tumorigenesis via enrichment in fecal and mucosal samples of cancer patients (Boleij et al., 2015; Haghi et al., 2019). ETBF encodes the metalloprotease Bacteroides Fragilis Toxin (BFT), which induces chronic intestinal inflammation and tissue damage in CRC by targeting intestinal cell tight junctions, cleaving E-cadherin and thus increasing inflammation and intestinal permeability (Cheng et al., 2020). Furthermore, Wnt/β-catenin and NF-κB signaling (Wu et al., 2003, 2004), as well as Th17 adaptive immunity, are activated in this process (Cheng et al., 2020; Kim et al., 2008). Mechanistically, ETBF infection may play an important role in stemness regulation via upregulation of epigenetic and transcriptional regulator levels in a toll-like receptor (TLR4)-dependent pathway, which promotes colorectal carcinogenesis both *in vitro* and *in vivo* (Liu et al., 2020).

Salmonella enterica

During *Salmonella* infection in mouse models and cells, activation of MAPK and AKT pathways is critical to sustain cellular transformation in gallbladder cancer (Scanu et al., 2015). A type 3 secretion system (T3SS) transfers into target cells effector proteins such as cyclomodulin-like protein Typhoid toxin and the multifunctional effector AvrA, both involved in triggering cancer via genotoxin-mediated mutagenesis. In addition, the cyclomodulin-like protein allows intracellular bacterial survival and favors dysbiosis (Del Bel Belluz et al., 2016), while AvrA promotes Wnt/β-catenin and JAK/STAT pathway activation, cell proliferation and differentiation, and enhanced acetyltransferase activity targeting p53, collectively driving cell-cycle arrest and apoptosis inhibition leading to tumorigenesis (Lu et al., 2017; Wu et al., 2010).

Nonbacterial cancer-associated microbes

In addition to bacteria, other kingdoms within the microbiome, including the virome, mycobiome, and parasitome, may also contribute to cancer. While these are exemplified below, more extensive descriptions of the important contributions of viruses (Liang and Bushman, 2021; Stern et al., 2019), parasites (Leung et al., 2018), and fungi (Dambuza and Brown, 2019; Galloway-Pena and Kontoyiannis, 2020) to health and disease, including cancer, are discussed elsewhere.

Viruses

Similar to the bacterial microbiome, the virome, composed of 98% bacteriophages and 2% eukaryotic viruses (Gregory et al., 2020), represents a complex ensemble of commensal and pathogenic viruses inhabiting multiple body sites that is established as early as 1 month after birth (Backhed et al., 2015; Liang et al., 2020). Recent studies have indicated that global virome signatures may serve as markers of cancer development. Viral metagenomic signatures have been detected in lung adenocarcinoma biopsies (Cai et al., 2021) and more than 100 tumor samples, including oral, breast, colon, and genitourinary cancers (Mollerup et al., 2019). Cantalupo et al. suggested that HPV, a known cause of cervical cancer and potentially other genitourinary cancers, is additionally correlated with tumor progression in head and neck cancers as well as bladder cancers (Cantalupo et al., 2018; IARC, 2009). In these cases, gene expression and mutational profiles indicate that HPV might drive tumorigenesis, yet the precise mechanisms of HPV involvement in cancer induction remain elusive.

In addition to eukaryotic viruses, bacteriophages may also act as modulators of cancer. For example, some bacteriophages may interact with cancer cells and downregulate the expression of integrins and other proteins involved in carcinogenesis and metastasis (Putra and Lyrawati, 2020). Other phages, of the families Syphoviridae and Myoviridae, were enriched in CRC patient feces. As the majority of those phages were temperate, it remains unclear whether they represent a true biological signal or are the result of temperate phage overrepresentation in genetic reference databases (Hannigan et al., 2018). Likewise, increased populations of multiple *Streptococcus*-specific bacteriophages and a *Vibrio*-inhabiting bacteriophage were detected in the gut of CRC patients as compared with controls (Nakatsu et al., 2018). Putatively, these bacteriophage alterations may initiate genetic exchange, enabling ecological adaptations and community networking within the host, thereby impacting cancer (Putra and Lyrawati, 2020). These associations notwithstanding, a direct effect of phages on carcinogenesis has yet to be shown. An elegant example of the potential transkingdom interplay among viruses, their bacterial associates, and eukaryotic cells, which may hold relevance to cancer, involved skin-associated *Staphylococcus epidermidis*, which activates the expression of endogenous skin retroviral components, and in turn sets off a commensal-specific T cell response promoting tissue repair (Lima-Junior et al., 2021). Whether similar microbial mechanisms will impact cancer-relevant processes will likely constitute exciting avenues of cancer-microbiome research in coming years.

Fungi

A dysbiotic mycobiome is increasingly suggested to associate with multiple pathologies including acute graft versus host disease (van der Velden et al., 2013), as well as oral cancer (Mukherjee et al., 2017) and CRC (Coker et al., 2019). Fourteen fungal biomarkers were associated with CRC and correlated with a bacterial dysbiosis including enrichment of the phylum Fusobacteria and the fungal genus *Malassezia*, in colorectal and pancreatic cancer, respectively (Aykut et al., 2019; Coker et al., 2019). Mechanistically, *Malassezia* migrates to the pancreas and activates the complement cascade via the binding of host mannose-biding lectins to fungal cell walls (Aykut et al., 2019). Recently, an outgrowth of *Candida albicans* was associated with, and suggested to be even predictive of, gastric cancers (Zhong et al., 2021). Several putative mechanisms may causally link *C. albicans* and cancer risk, including production of nitrosamines, which are known to alter cell proliferation in oral cancers (Sanjaya et al., 2011), induction of immune modulation via TNFα and IL-18, and promotion of tumor cell adhesion to epithelial cells (Ramirez-Garcia et al., 2013). These merit future mechanistic studies. The mycobiome and bacterial microbiome may also influence each other and their host in homeostatic and disease scenarios, as exemplified by fungal recovery after the usage of antibiotics (Seelbinder et al., 2020), and fungal-bacterial interactions driving differential tumor responses to radiotherapy

in mouse breast cancer and melanoma models (Shiao et al., 2021). These interactions have been reviewed elsewhere (Kapitan et al., 2019); however, many aspects related to potential causal mycobiome impacts on cancer remain mechanistically elusive.

Parasites

Outside of the known parasitic carcinogens confirmed by the IARC (IARC, 2009), the parasitome has not yet been wholly described as influencing cancer development. Parasitic microbiome members may indirectly modulate other microbiome kingdoms by inducing cancer-promoting dysbiosis. For example, Gram-negative bacteria such as *Salmonella*, *Klebsiella*, and *E. coli* are more prevalent in patients with bladder cancer associated with *S. haematobium* (Mostafa et al., 1994, 1999). In addition, parasites may directly modulate cancer development through induction of immune modulation. For example, multiple parasites can induce both innate and adaptive immune responses via regulation of TLRs and inflammasomes, in addition to inducing T regulatory cell activities (Leung et al., 2018). Inversely, parasites may induce beneficial effects of antitumoral action and are even suggested as adjuvants in cancer therapy (Callejas et al., 2018). These and other parasitic contributions to cancer merit further mechanistic exploration.

Microbiome community alterations and cancer

In addition to discrete pathogenic or commensal members of the microbiome directly contributing to cancer development, more global disturbances in the commensal population of bacteria, termed dysbiosis, are increasingly suggested to contribute to cancer development through a variety of community-driven mechanisms involving bacterial-bacterial interactions (Wilkins et al., 2019). The oral cavity, as an example, features population-level shifts from exposure to smoking or dietary changes that can induce niche changes (such as pH alterations), in turn impacting commensal inhabitants and their secreted byproducts (Burne and Marquis, 2000; Mukherjee et al., 2021; Xu et al., 2015). Oral cavity dysbiosis has been correlated with the onset of dental caries, periodontitis (Kolenbrander et al., 2010; Marsh, 2003), and oropharyngeal cancers like OSCC (Yang et al., 2018), and is mediated, in part, by expansion of opportunistic pathogens like *Streptococcus mutans* (de Soet et al., 2000), *F. nucleatum*, and *P. gingivalis* (Binder Gallimidi et al., 2015; Schmidt et al., 2014). Likewise, microbiome shifts within the lower GI tract correlate with GI cancer and CRC (Levy et al., 2017). For instance, a healthy gut microbiome is generally populated by species representative of *Lactobacilli*, *Bacteroides*, and *Bifidobacterium* (Nakatsu et al., 2015). CRCs are marked by an overrepresentation of *Fusobacterium*, *Porphyromonas*, *Parvimonas*, *Peptostreptococcus,* and *Gemella* spp., indicative of microbial dysbiosis (Nakatsu et al., 2015; Wirbel et al., 2019).

This dysbiotic correlation becomes further complicated when considering different stages of tumorigenesis. Nakatsu et al. reported that as a colorectal neoplasm progresses from an adenoma to a carcinoma, microhabitats are formed that each have their own metacommunity of microorganisms. In addition, colorectal lesions feature a disease-specific microbiome configuration as compared with the adjacent mucosa, including an expanded representation of oral-associated microbes such as Fusobacteria (Nakatsu et al., 2015). As another example, the healthy vaginal microbiome is characterized by a low diversity of *Lactobacillus*-dominated commensal organisms. An outgrowth of anaerobic bacteria contributes to bacterial vaginosis (BV), whereas expansion of members of the vaginal mycobiome, such as *Candida albicans*, may cause vaginitis characterized by a local host inflammatory reaction (Lev-Sagie et al., 2019). In addition to HPV (IARC, 2009), BV correlates with both premalignant lesions of the cervix (Barrington et al., 1997) as well as intraepithelial neoplasia (Guijon et al., 1992). A recent meta-analysis additionally determined BV to be a cofactor in HPV-positive women with cervical cancer, signaling that this dysbiosis likely plays a direct and additional role in the onset of cervical cancer (Liang et al., 2019). Of note, the above dysbiosis-cancer correlations mostly constitute an associative, rather than a causal relationship. As such, it is unclear as to whether the population shift causes carcinogenesis or rather is a consequence of the emerging tumors. This important distinction will be at the focus of cancer-microbiome research in the coming decade.

INTRATUMORAL MICROBIOMES

In addition to the *bona fide* microbiome mucosal niches (GI tract, respiratory tract, genitourinary tract, and skin), it has been recently suggested that intratumoral microbes (also termed the "tumor microbiome") (Riquelme et al., 2019; Sepich-Poore et al., 2021) may constitute distinct low-biomass ecosystems. These may impact the TME, including inflammatory mediators such as tissue resident and peripherally recruited immune cells (myeloid cells; T, B, and NK cells), fibroblasts, endothelial cells, adipocytes, and pericytes (Quail and Joyce, 2013) (Balkwill et al., 2012). An analysis of more than 1,000 tumor samples of seven cancer types, and adjacent noncancerous tissues, identified tumor-type–specific microbiomes composed mostly of intracellular bacteria. As an example, *Klebsiella pneumoniae* was identified in samples from lung and pancreatic cancer, while other common denominators (*Enterobacter cloacae*, *Citrobacter freundii*, *Enterobacter asburiae*, and *Fusobacterium*) were concomitantly associated with pancreatic cancer as well as breast cancer (Nejman et al., 2020). It is still uncertain whether these microbes constitute a predetermined niche or rather represent a transient stochastic colonization. As an example, cancer-associated microbes found at the intrapancreatic tumor environment may reach the pancreas via peri-intestinal translocation through the pancreatic duct (Del Castillo et al., 2019). Gut epithelial barrier damage may also influence the intratumoral microbiome population, in which hematogenous microbial spread may allow for tumor tissue colonization. Recently, intratumoral CRC-associated *E. coli* was shown to migrate to the liver after gut vascular barrier disruption, where it then primed the liver microenvironment through recruitment of innate and inflammatory immune cells to directly promote metastasis (Bertocchi et al., 2021). Furthermore, oncogenic bacteria ascendant from the cervix may reach and colonize the uterus and ovaries during carcinogenesis (Laniewski et al., 2020).

Functionally, the contribution of these intratumoral microbes to the TME and tumor-related pathogenesis is only beginning to be explored and merits further mechanistic investigation. Predictive models suggest that bacteria present in tumors may likely

upregulate pathways specific for particular body sites. As an example, bacteria enriched in lung carcinomas may potentially possess an ability to metabolize cigarette-associated metabolites (Nejman et al., 2020). In PDAC, the tumor microbiome inactivated the chemotherapeutic gemcitabine via a specific isoform of the enzyme cytidine deaminase produced by Gammaproteobacteria (Geller et al., 2017). Depletion of the microbiome either in germ-free mice or upon antibiotic treatment facilitated immunogenic TME reprogramming with reduced myeloid-derived suppressor cell (MDSC) infiltration, increased Th1-type CD4+ T cell polarization, and activation of cytotoxic CD8+ T cells that significantly reduced carcinogenesis and increased the efficacy of immunotherapy (Pushalkar et al., 2018).

MECHANISMS OF MICROBIAL IMPACTS ON CANCER

A variety of contact-dependent, contact-independent, and immunological mechanisms drive the intricate host-microbe interactions that result in microbiome-induced cancer modulation. Of note, individual microorganisms can manifest pleiotropic interactions impacting tumorigenesis that may combine presentation and secretion of virulence factors, physical binding-induced signaling, and immune cell recruitment, collectively contributing to carcinogenic influences. Understanding these mechanisms is critical in harnessing these interactions as putative cancer diagnostics and treatments.

Contact-dependent interactions

A surfeit of mechanisms extrapolated by indigenous commensals and invasive pathogens may impact cancer-related processes through direct interactions with targeted host cells. For example, in the stomach mucosa, injection of *H. pylori* CagA into target epithelial cells directly interacts with E-cadherin, disrupting the E-cadherin/β-catenin association, allowing for nuclear β-catenin accumulation and ultimately inducing downstream intestinal differentiation markers that lead to premalignant intestinal metaplasia (Murata-Kamiya et al., 2007). Similarly, injection of AvrA of *S. enterica* upregulates β-catenin pathways, activating STAT3 signaling and inflammation, as well as EMT-inducing transcription factors (Lu et al., 2017). A direct interaction between *F. nucleatum* and E-cadherin also involves binding of FadA (Rubinstein et al., 2013), which can lead to DNA damage, epithelial cell proliferation, acquisition of stemness, and loss of cellular polarity via increased expression of downstream E-cadherin/β-catenin–modulated transcription factors (Guo et al., 2020; Rubinstein et al., 2013). Bacteria can also target the cell cycle via contact-dependent release of different classes of cyclomodulins to stimulate the release of reactive oxygen and nitrogen species within host cells. The cytolethal distending toxin (CDT), one class of cyclomodulin generated in *Escherichia*, *Helicobacter*, and *Salmonella*, binds to an unknown receptor on host cells to internalize and ultimately inhibit cellular proliferation via induction of DNA damage (De Rycke and Oswald, 2001). Such cell-cycle and DNA damage modulation may contribute to cancer initiation.

Contact-independent interactions

Microbes can also indirectly influence carcinogenesis via remote production and metabolism of bioactive biomolecules and OMVs, which may reach tumor and metastasis sites via the systemic circulation. Secreted metabolites, fermentation products, and catabolites are active across different tumor niches (Rossi et al., 2020). For example, lipopolysaccharide toxin (LPS) and acetate can facilitate carcinogenesis via promotion of EMT and angiogenesis (Rossi et al., 2020), whereas the diamine cadaverine reverts EMT and inhibits cellular movements and invasion in *in vitro* assays (Kovacs et al., 2019). Host-secreted metabolites that undergo microbiome metabolism, such as the secondary bile acids (sBAs) deoxycholic acid (DCA), and lithocholic acid (LCA), have been implicated in the onset of distinct cancers such as CRC and hepatocellular carcinoma (Rossi et al., 2020). Likewise, a higher concentration of the genotoxic microbe-produced compound hydrogen sulfide was found in CRC biopsies, as compared with adjacent healthy tissues. *In vitro*, hydrogen sulfide modulated cell energy metabolism and proliferation (Blachier et al., 2021). Microbially modulated metabolites can originate from diet. For example, fatty diets can lead to the increased production of bile acids such as Tyr-Chol by *Clostridium* spp. in Crohn's disease, which may contribute to inflammation-induced tumorigenesis in this clinical context (Quinn et al., 2020). On the other hand, conversion of primary to secondary bile acids in mice by commensal bacteria can favorably impact the outcome of subcutaneous tumors and liver metastases, by inducing a CXCR6+ natural killer T (NKT) cell response (Ma et al., 2018).

In some instances, the impacts of microbiome-modulated metabolites on cancer development may be niche-specific. Kadosh et al. recently showed that a microbially derived gallic acid was involved in the enigmatic relationship of p53 mutations in different intestinal niches that led either to tumor suppression in the proximal gut or a malignant cellular transformation in the distal gut (Kadosh et al., 2020). The microbiome is also able to metabolize host hormones that can affect carcinogenesis. For instance, commensals, including *E. coli* and *Enterobacter* species, deconjugate estrogen metabolites, which return in their bioactive forms into the host portal circulation through the enterohepatic circulation, thereby facilitating carcinogenesis of estrogen-mediated cancers, such as breast and prostate cancer (Kwa et al., 2016; Parida and Sharma, 2019). In male individuals, the gut and urinary microbiome can increase the risk of prostate cancer via generation of intermediate oxyandrogens from glucocorticoids (Ridlon et al., 2013; Sha et al., 2020).

An important bacterial delivery system that is involved in carcinogenic processes is composed of bacterial extracellular OMVs, mainly produced by Gram-negative bacteria. This strategy allows bacteria to laterally transfer genetic material, immunomodulatory molecules, virulence factors, and toxins into systemic circulation, while its membrane encapsulation serves as decoy for antibodies and commensal defensins. Importantly, many virulence factors, including oncogenic molecules such as CagA, VacA, BFT, and potentially colibactin, can be transported via OMV and contribute to tumor promotion at remote niches (Canas et al., 2016; Chmiela et al., 2018; Ricci et al., 2020; Zakharzhevskaya et al., 2017a, 2017b). For example, *H. pylori* OMVs carrying CagA alter DNA binding to histones within the nucleosome and disrupt epithelial cell binding, which causes a transition toward an invasive mesenchymal cell that can then progress to cancer (Chmiela et al., 2018; Turkina et al., 2015).

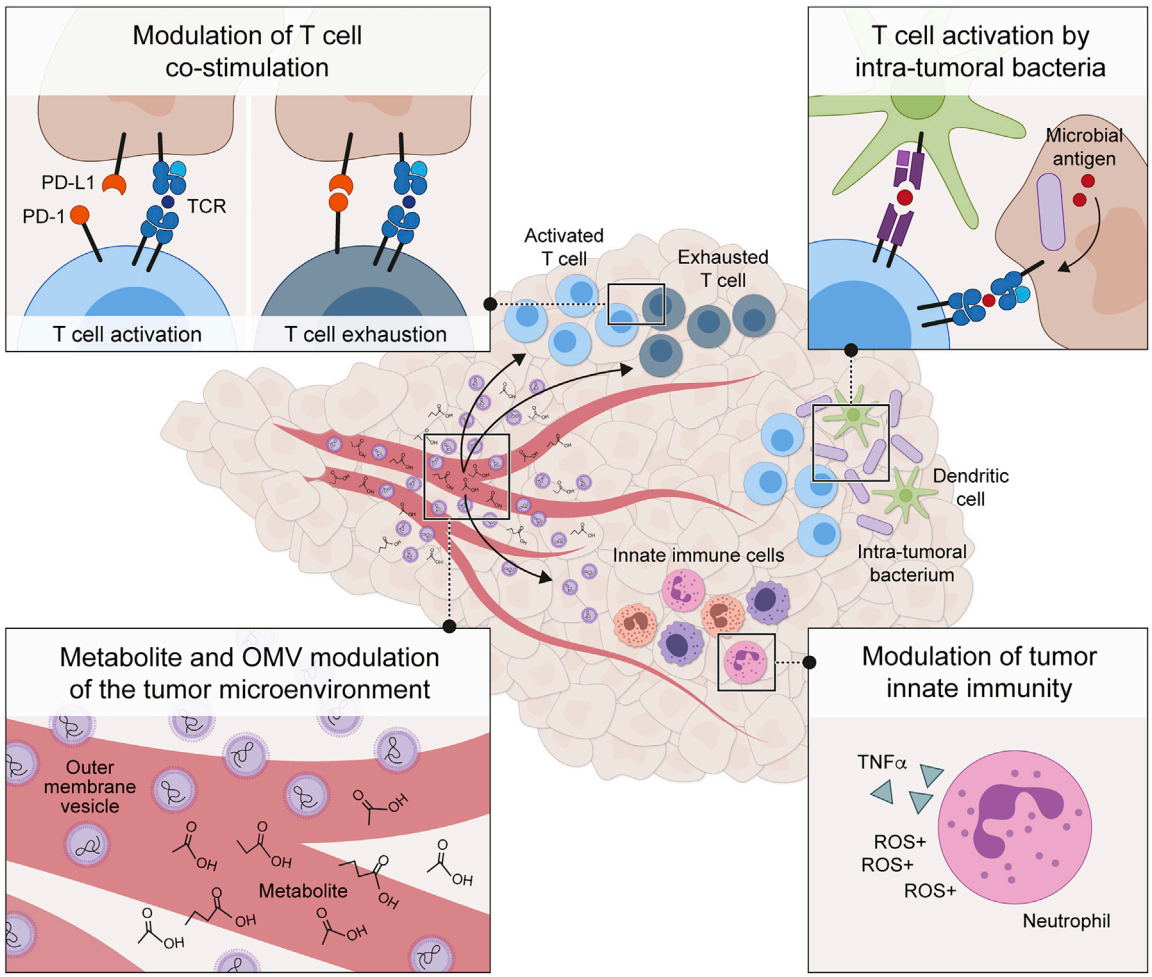

Figure 3. Microbiome modulation of the tumor immune microenvironment

Exemplified are means by which microbiome-secreted metabolites, cargo-carrying OMVs, or intratumoral bacteria may induce a complex array of immunomodulatory actions modulating tumor growth and immunosuppressive environments within and around the tumor. Microbial secreted moieties can impact the TME innate immune response by modulating attraction and activation of innate immune cells such as neutrophils, producing TNFα and reactive oxygen species (ROS+) to combat tumor cells. Microbial metabolites and OMVs can also impact the adaptive immune response. By modulating T cell co-stimulation, they impact TME T cell activation and exhaustion, directly impacting ICI efficacy. In addition, peptide fragments from intracellular bacteria can be directly presented on the tumor cell surface or on "professional" antigen-presenting cells by HLA, thereby driving T cell activation and potentially cancer immune reactivity.

In addition, commensal microbes at mucosal sites can indirectly impact microbial contributions to cancer by opposing pathogen and pathobiont colonization and host invasion (Mullineaux-Sanders et al., 2018). Such intercommensal and host interactions occur across body niches by inducing colonization resistance via mucosal barrier fortification, modulation of mucosal antipathogenic immune responses, and alteration by biophysical properties, such as acidity, oxygenation, and iron availability (Leshem et al., 2020).

Immune interactions

Immune-microbe interactions in cancer occur either at mucosal surfaces, systemically via the action of microbial metabolites and OMVs, or locally within lymphoid organs or the TME itself. Local and remote microbial signals may impact both innate and adaptive immune responses (as exemplified in Figure 3), leading to modulation of systemic or TME immunity and immunosurveillance. Microbiome-derived metabolites can reach remote tumor entities through the systemic circulation, where they may stimulate antitumoral or carcinogenic innate immune responses. For example, evolutionary conserved microbe-associated molecular patterns (MAMPs) originating from commensals or pathogens (Chu and Mazmanian, 2013; Mogensen, 2009) may be systemically sensed by the innate immune system via pattern recognition receptors (PRRs) such as TLR and NOD-like receptors, leading to innate immune responses and innate instruction of the adaptive immune response. Bacterial MAMPs can boost antitumor immunity via augmented TLR signaling and by serving as cancer vaccine adjuvants (Fessler et al., 2019; Luchner et al., 2021). Other examples of gut microbiome–modulated bioactive metabolites impacting tumor innate immunity include sBAs that control tumor progression via modulation of hepatic NKT cells in the hepatocellular carcinoma TME (Ma et al., 2018).

Microbial-derived signals can also modify the tumor-associated adaptive immune response. T cell exhaustion within the

TME, an important phenomenon driving an immune failure to clear tumors, is regulated, among several mechanisms, by a T cell–expressed aryl hydrocarbon receptor (Liu et al., 2021b). This receptor, in turn, is activated by 5-hydroxytryptophan, a by-product of gut microbiome-induced tryptophan metabolism, thereby leading to microbial promotion of immune-mediated tumorigenesis (Yano et al., 2015). Conversely, the gut commensals *Erythrobacter ramosus* and *B. fragilis* located in the ileum facilitate the induction of follicular T helper (Tfh) cells via activation of dendritic cells and release of IL-1 and IL-12 under conditions of oxaliplatin-induced cell death of gut enterocytes. This amplifies the maturation of B cells in tertiary lymphoid structures and enhances antibody-dependent cytotoxicity against tumor cells and the efficacy of ICI in tumor mouse models (Roberti et al., 2020). However, the exact mode of microbe-immune cell interaction in this complex scenario has yet to be fully determined. Similarly, the diet-derived and microbially modulated SCFAs butyrate and pentanoate enhance tumor killing of chimeric antigen receptor (CAR)-T cells targeted against ROR1-expressing tumors in mice by epigenetic modulations of CTL-effector molecule expression (Luu et al., 2021).

OMVs represent another systemic conduit of microbial-derived cargos impacting tumor immunity. For example, non-toxigenic *B. fragilis* OMVs carry the MAMP polysaccharide A (PSA) (Shen et al., 2012), which features local (Round et al., 2011) (Shen et al., 2012) and systemic anti-inflammatory capacities (Ramakrishna et al., 2019). Similar PSA effects in cancer immunity have been recently suggested (Lee et al., 2021; Mazmanian et al., 2008) to be protective for CRC cell proliferation and suppression of EMT via TLR2 signaling (Sittipo et al., 2018).

In addition to the above described remote immunogenic effects, commensal microbes residing in the TME can induce a complex array of immunomodulatory actions, including facilitating predominantly immunosuppressive environments, or conversely stimulating innate immune cells via PRRs to activate proinflammatory cytokine production, thereby driving the influx of immune cells with consequent antigen presentation for antitumoral immune function (Zitvogel et al., 2016). In some cases, the same cancer-modulating microbe may induce both pro- and anti-inflammatory reactions. As an example, adherence of *F. nucleatum* to CRC cells via FadA stimulates the release of inflammatory factors, such as NF-κB, IL-6, IL-8, IL-10, and IL-18 (Rubinstein et al., 2013), and increases the infiltration of inflammatory cells including macrophages, dendritic cells, and granulocytes. Overall, this creates a proinflammatory microenvironment that further facilitates tumorigenesis (Kostic et al., 2013). In addition, *F. nucleatum* can create an immunosuppressive TME, reflected by an inverse correlation of *F. nucleatum* abundance and CD3+ T cell density in CRC (Mima et al., 2015). Induction of T cell apoptosis through Fap2 and RadD, or activation of the inhibitory T cell receptor TIGIT through Fap2, may contribute to immunosuppression (Gur et al., 2015; Kaplan et al., 2010). In addition, *F. nucleatum* can release formylmethionyl-leucyl-phenylalanine, which leads to recruitment of MDSCs and can regulate immune responses by suppressing $CD4^+$ T helper cell function (Kostic et al., 2013). Interestingly, human melanoma cells express a repertoire of peptide fragments presented on the tumor cell surface by HLA, derived from intratumoral bacteria including *F. nucleatum*. This microbial peptide antigen presentation is capable of eliciting T cell recognition and immune reactivity (Kalaora et al., 2021), suggesting that, in some contexts, tumor-residing microbes may directly impact TME immune activation. Microbiome-cancer immunotherapy interactions and their potential impacts on treatment effectiveness are further described below. The scope of potential immune effects on tumor growth and metastasis formation merits further studies.

MICROBIOME-ASSISTED CANCER DIAGNOSIS

With the deepening realization that the microbiome may impact cancer pathogenesis, utilization of microbiome-derived personalized data constitutes an exciting avenue of research aimed at integrating microbiome readouts into the precision oncology setting, as is depicted in Figure 4.

Recent studies have provided evidence that unique microbial DNA and RNA signatures can be detected in blood samples. Stringent filtering criteria in more than 10,000 screened patients identified microbial plasma signature predictive of cancer types, that could be further differentiated from their respective healthy tissue profiles (Poore et al., 2020). In addition to cancer diagnosis, deciphering key microbiome signatures within different stages of cancer progression may offer possibilities for treatment stratification and metastasis surveillance. Indeed, recent studies identified *F. nucleatum* and an upregulation of its tumor cell binding partner, Gal-GalNAc, in breast tumors and metastatic sites alike (Parhi et al., 2020). Similarly, *F. nucleatum* signatures were detected in CRC metastases and suggested to contribute to their development (Chen et al., 2020).

Profiling the microbiome may also offer possibilities for a prognostic assessment. Notably, a distinct intratumoral microbial diversity and composition supported the short- and long-term survival assessment in PDAC patients (Riquelme et al., 2019). Mining microbiome data may also be used to anticipate adverse reactions to cancer immuno- and chemotherapy. As an example, mucositis of the GI tract and oral mucosa constitutes a major complication of chemotherapy and is associated with microbial dysbiosis (van Vliet et al., 2010). In oral mucositis, dysbiosis is marked by a decrease in multiple commensal *Streptococcus* and *Prevotella* species and an increase in the proinflammatory *F. nucleatum*. These dysbiotic signatures were further utilized to gauge oral mucositis events in melanoma patients undergoing chemotherapy (Hong et al., 2019; Laheij et al., 2019).

MICROBIOME IN CANCER TREATMENT

Beyond the above diagnostic uses of microbiome data, exploring microbiome influences on cancer therapy responsiveness constitutes one of the most exciting and potentially translational aspects of cancer-microbiome research, and may lead to data-driven optimization of the oncologic therapeutic decision-making process.

Immunotherapy

Immune-based anticancer treatments refer to a spectrum of therapeutic approaches designed to empower a patient's immune system or utilize third-party immune components to attack

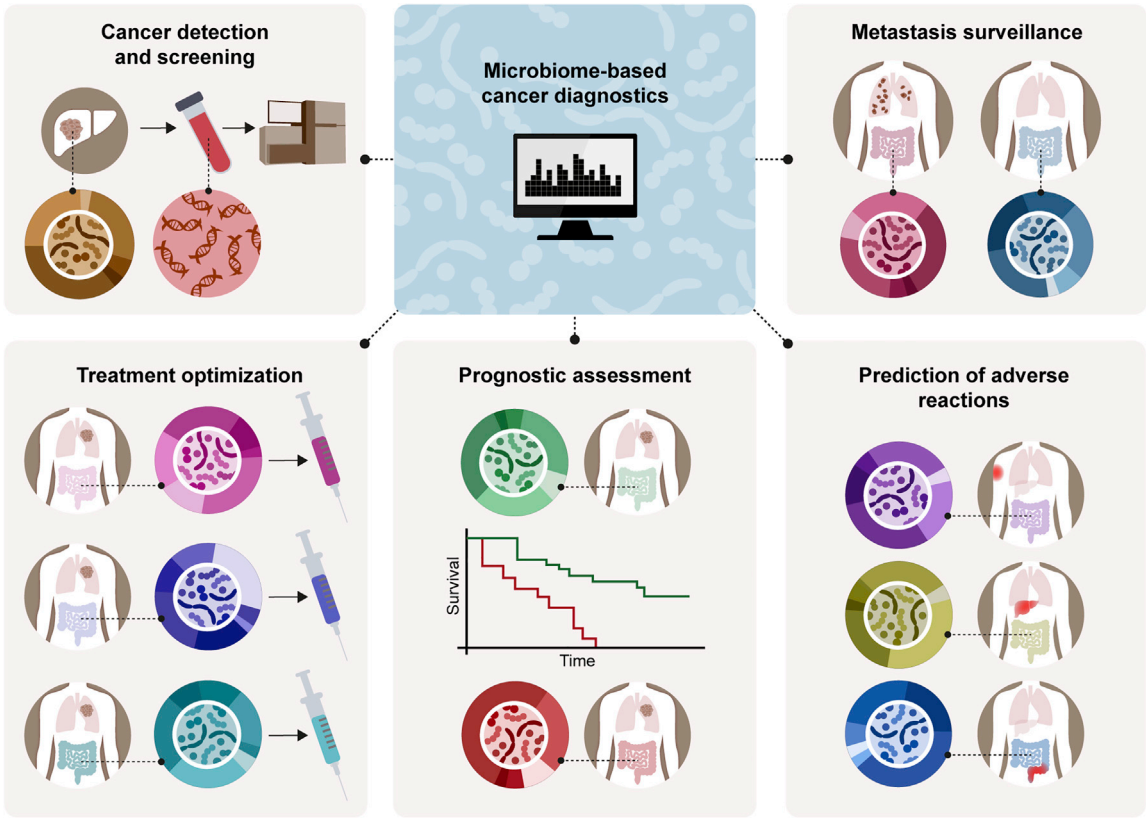

Figure 4. Utilization of microbiome data in cancer diagnosis and patient stratification
Data generated by microbiome analysis may facilitate the development of new cancer diagnostic capacities, including cancer detection by identification of microbial DNA and RNA in peripheral blood, surveying for micro-metastatic progression in cancers, assessing prognosis, tailoring treatment regimens to the individual, and utilizing artificial intelligence algorithms in predicting patient treatment responses and risk of adverse effects.

cancer. This approach is currently spearheaded by interventions targeting negative regulators of T cell activation, called "immune checkpoints," which are often "hijacked" by the tumor in inducing an immune-privileged TME. Checkpoint inhibitors, such as antibodies against programmed cell death protein 1 (PD-1) or its ligand PD-L1 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), can block the interaction of T cells with their suppressive cognate ligands on tumor or stromal cells (Pardoll, 2012; Ribas and Wolchok, 2018) to unleash an antitumor immune response. Effects of this intervention, noted in a minority of patients, range from complete remission in rare cases to significant life prolongation even in metastatic cancers (metastatic melanoma, non–small-cell lung cancer, Hodgkin lymphoma, and renal cell carcinoma as examples). In 2015, two mouse studies showed that members of the commensal gut microbiome including *Bifidobacterium* spp. were capable of enhancing the antitumor efficacy of PD-L1 checkpoint blockade (Sivan et al., 2015), whereas *Bacteroides thetaiotaomicron* and *B. fragilis* were associated with enhanced CTLA-4 inhibitor efficacy (Sivan et al., 2015; Vétizou et al., 2015). In addition, the antitumor efficacies of PD-1/L1-targeting therapies were associated with multiple bacteria, including *Akkermansia*, *Faecalibacterium*, *Clostridiales*, and *Bifidobacterium* spp. (Gopalakrishnan et al., 2018a; Matson et al., 2018; Routy et al., 2018b). In germ-free mice colonized with rationally selected, IFNγ-inducing bacterial strains, efficacy of ICIs and antitumor T cell response was significantly enhanced (Tanoue et al., 2019). These effects were partially ascribed to impacts mediated by microbial metabolites such as the SCFAs butyrate and propionate. However, these effects remain conflicting in some contexts. For instance, high fecal SCFA levels have been associated with longer progression-free survival or increased antitumor responses, whereas high systemic levels were associated with poorer treatment responses (Hayase and Jenq, 2021). Butyrate may also limit the capacity of dendritic cells to induce tumor-specific T cells and memory T cells, thereby restraining the efficacy of anti-CTLA-4 ICI (Coutzac et al., 2020).

Other microbial metabolites also impact ICI. For example, *Bifidobacterium pseudolongum*-generated inosine enhances ICIs through activation of A_{2A} receptors on T cells (Mager et al., 2020). Alternative routes of microbe-host interactions in cancer immunotherapy include direct stimulation of dendritic cells in lymph nodes by *Akkermansia muciniphila* to increase the antitumor efficacy of ICIs in an IL-12–dependent manner (Routy et al., 2018b) or by *Bacteroides* spp. via induction of Th1 and CD8+ T cell antitumor immune responses (Routy et al., 2018b; Vétizou et al., 2015). Therapeutic exploitation of these microbiome impacts on cancer immunotherapy by antibiotics administration, microbial transfer, or metabolite supplementation are further described below.

Chemotherapy

Commensal microbes can modulate chemotherapy effectiveness. For example, *E. coli* may modulate the efficacy of two anticancer drugs, gemcitabine and CB1954, by inducing resistance and activating cytotoxicity in tumors, respectively. Gemcitabine has also been shown to be metabolized by bacteria present in human PDAC, an effect correlating with intratumoral LPS abundance and overcome by antibiotic treatment (Geller et al., 2017). More than a dozen other anticancer drugs were found to be potentially modulated by bacteria *in vitro* (Lehouritis et al., 2015). Conversely in mice, oxaliplatin and cyclophosphamide are less efficient in inhibiting tumor growth in germ-free mice or mice treated with broad-spectrum antibiotics. Upon antibiotics-mediated commensal depletion, tumor-infiltrating myeloid cells responded poorly to CpG-oligonucleotide tumor immunotherapy with lower TNF production, or to oxaliplatin therapy with reduced production of reactive oxygen species and deficient cytotoxicity (Iida et al., 2013). In addition, chemotherapy-associated gut barrier impairment enables gut commensal translocation to secondary lymphoid organs, where they elicit systemic induction of Th17-type tumor antigen-specific CTLs in mouse models (Daillère et al., 2016). Antibiotic treatment prevents such commensal gut translocation and associated T cell polarization, thereby attenuating the tumoricidal activity of chemotherapy (Viaud et al., 2013).

In addition to microbial impacts on chemotherapy effectiveness, chemotherapy and associated mucosal damage can impact the gut microbiome composition. Even before the widespread use of NGS techniques, culture-based methods provided evidence that chemotherapeutic agents such as 5-fluorouracil (5-FU) can modulate the oral and fecal microbiome of laboratory animals with an expansion of Gram-negative anaerobes (Von Bultzingslowen et al., 2003). These findings were later expanded by *16S rRNA* sequencing, revealing a decrease in *Eubacterium* and *Ruminococcus* spp. (Le Bastard et al., 2018). Irinotecan treatment was associated with specific gut microbiome dysbiotic configurations and expanded expression of microbial β-glucuronidases (Guthrie et al., 2017). Likewise, patients receiving a myeloablative conditioning therapy for non-Hodgkin's lymphoma featured an expansion of Enterobacteriaceae and Enterococcaceae and a loss of Ruminococcaceae, Lachnospiraceae, and *Bifidobacterium* spp. (Montassier et al., 2015), while allogeneic hematopoietic cell transplantation (allo-HCT) and immune cell reconstitution has been associated with an expansion of the gut commensals *Faecalibacterium*, *Ruminococcus*, and *Akkermansia* spp. (Schluter et al., 2020). The roles of these chemotherapy-induced microbial alterations in impacting tumorigenesis, treatment responses, and chemotherapy-induced adverse effects merit further studies.

Radiotherapy

The interplay between radiotherapy and dysbiosis is reviewed elsewhere (Liu et al., 2021a). Post-radiotherapy, gut microbiome dysbiosis is marked by a decreased abundance of commensal *Bifidobacterium*, *Faecalibacterium*, and *Clostridium* spp. and an increased abundance of *Bacteroides* and *Enterococcus* spp. (Liu et al., 2021a; Touchefeu et al., 2014). Patients receiving pelvic radiotherapy feature a 3% increase in gut Fusobacteria taxa (Nam et al., 2013), bearing potential important consequences given the tumor-promoting capacities of some members of this phylum. Beyond those associations, mouse studies demonstrated that oral vancomycin-induced depletion of Gram-positive gut commensals was correlated with enhanced radiotherapy effectiveness in melanoma, lung, and cervical cancer models, potentially mediated through IFNγ and CD8 T cell–dependent mechanisms (Uribe-Herranz et al., 2020). Conversely in a mouse breast cancer radiation treatment model, antibiotic-induced commensal depletion, including the order *Clostridiales*, led to an intestinal *Saccharomyces* expansion promoting a macrophage-mediated protumoral response (Shiao et al., 2021). The gut microbiome may additionally modulate radiotherapy toxicity, namely radiation enteritis driven by radiation-induced epithelial inflammatory damage, by translocating through the impaired gut barrier, further contributing to an uncontrolled intestinal immune response and tissue damage (Al-Qadami et al., 2019). Likewise, distinct microbiome changes are associated with severe diarrhea in patients receiving a variety of localized radiotherapies (Nam et al., 2013; Wang et al., 2015).

MICROBIOME MODULATION TOWARD IMPROVED CANCER THERAPY

A unique feature of the microbiome, regarded by many as a "second genome," is that in contrast to the human genome, it is amenable to modulation. Such rational microbiome-altering interventions may potentially evolve into treatment of cancer or its complications. In the following section, we exemplify such experimental methods and their potential uses in cancer treatment (Figure 5). An overview of currently running clinical studies that utilize microbiome interventions to leverage cancer therapy is provided in Table 1.

Microbiome transplantation

Replacing a disease-associated microbiome with a healthy configuration through fecal microbiome transplantation (FMT) is highly effective in the treatment of recurrent *Clostridioides difficile* infection (van Nood et al., 2013) and possibly in some cases of ulcerative colitis (Paramsothy et al., 2017). Recently, vaginal microbiome transplantation has shown promising preliminary results in treating intractable BV (Lev-Sagie et al., 2019). In the cancer context, transferring patient fecal samples into tumor-harboring germ-free or antibiotics-treated mice treated with ICIs (Zitvogel et al., 2018) demonstrated a causal role of specific microbiome configurations as drivers of improved efficacy of immunotherapy. Likewise, FMT from patients who featured a favorable response to ICI ("responders") into tumor-harboring germ-free mice could transfer such ICI responsiveness to recipient mice, while recipient mice transferred with "non-responder" microbiomes failed to respond to ICI (Gopalakrishnan et al., 2018b; Routy et al., 2018a). Recently, two first-in-human clinical trials demonstrated that FMT from ICI-responding melanoma patients into ICI-resistant melanoma patients reversed the ICI nonresponsiveness in a subset of FMT recipients (Baruch et al., 2021; Davar et al., 2021). Response in these trials was partially dependent on donor selection and successful engraftment of the donor material in the recipient GI tract. With these initial results notwithstanding, several clinical, regulatory, and scientific uncertainties, such as those related to effective donor

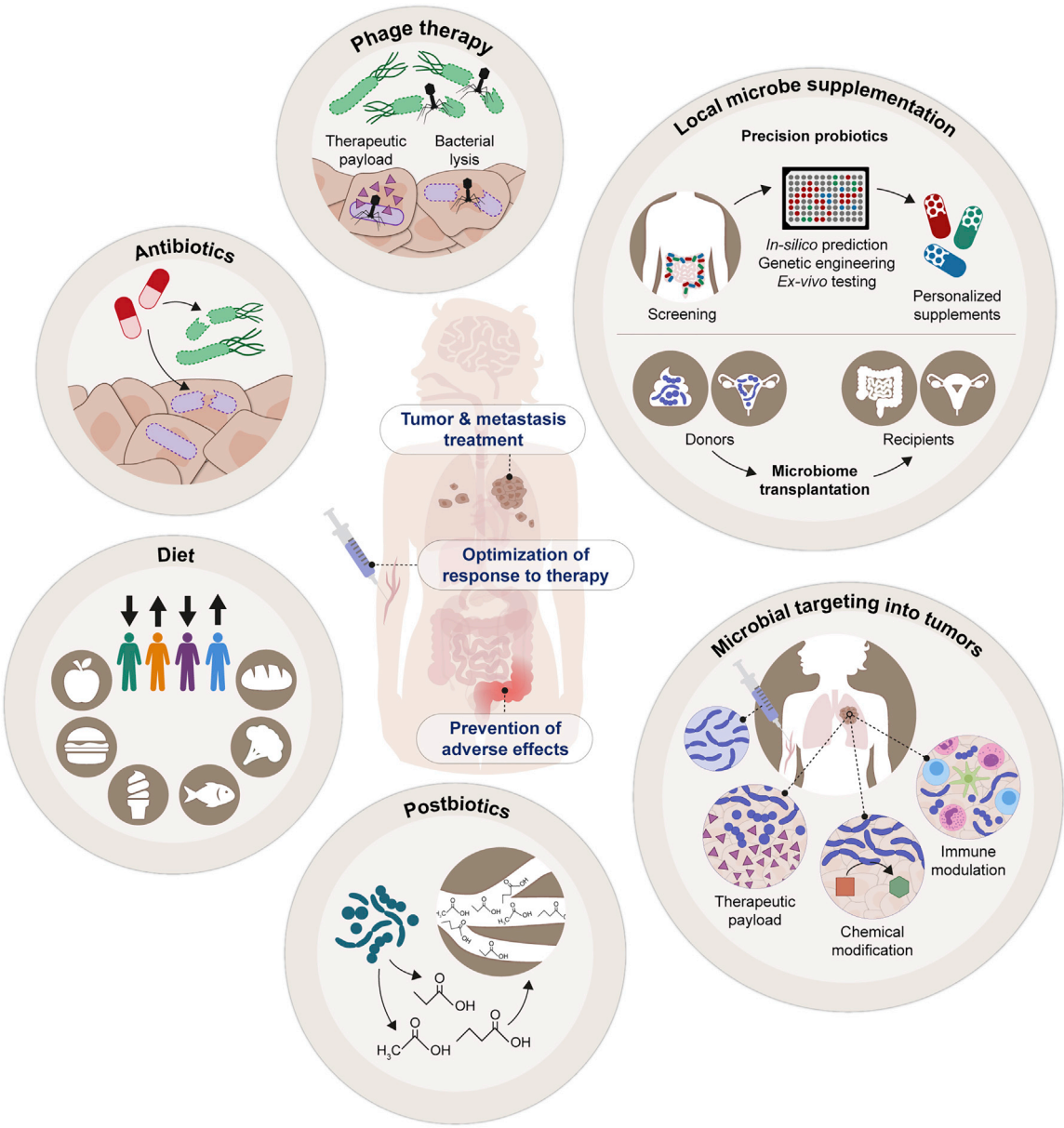

Figure 5. Microbiome modulation in cancer treatment

Exemplified are modalities potentially enabling rational microbiome manipulation contributing to cancer treatment. Data-driven dietary interventions may be harnessed to the individual and cancer type to induce reproducible and cancer-abating microbiome changes. Precision probiotics, consisting of gut-colonizing commensals whose functions are characterized through *ex vivo* and *in silico* prediction pipelines, may allow better colonization and host impacts. Whole community microbiome transfers, such as fecal and vaginal microbiome transplantation, may enable the replacement of a patient's microbiome with an anti-tumoral microbiome configuration. Mechanistic understanding of microbial factors impacting cancer and its treatment will allow for the development of "postbiotic" therapies, composed of supplementation of discrete and well-defined bioactive molecules rather than the microbes generating them. Targeted eradication of cancer-promoting microbes could be achieved, in a minority of cases, by antibiotics. An alternative approach consists of bacteriophage cocktails targeting commensals or intratumoral bacteria, while bearing minimal impacts on the surrounding microbial ecosystem. Alternatively, bacteriophage affinity to intratumoral bacteria could be harnessed toward targeted release of therapeutics in the tumor microenvironment. Likewise, systemic administration of tumor-attracting bacteria may elicit local immune responses and tumor-specific protective immunity, or enable tumor microenvironment alteration through microbial metabolic activity or through local release of therapeutic payloads.

and recipient selection, bowel preparation, and engraftment procedures, need to be addressed before this approach can be routinely adopted. Moreover, the FMT drivers of such clinical effects, including bacteria, phages, or microbial metabolites, remain elusive to date and merit large-scale, prospective clinical trials.

Probiotics

Over-the-counter probiotic preparations are widely used by the general public but necessitate additional rigorous non–industry-funded research in assessing their effectiveness, colonization capacity, and possible adverse effects (Suez et al., 2019). Probiotic preparations have been suggested to impact cancer

Table 1. Overview of current clinical studies investigating the effects of microbiome modulations in cancer therapies

Intervention	Cancer therapy	Cancer entity	Study phase	Endpoint	Reference
Fecal microbiota transfer (FMT)	Immune checkpoint inhibition	NSCLC, melanoma	Phase II	Therapy response	NCT04951583
	Immune checkpoint inhibition	Prostate cancer	Phase II	Therapy response	NCT04116775
	Immune checkpoint inhibition	CRC	Phase I	Therapy response	NCT04729322
	Immune checkpoint inhibition	RCC	Phase II	Therapy response	NCT04758507
	Immune checkpoint inhibition	Melanoma, NSCLC, GU cancer	Phase I	Toxicity	NCT03819296
	Immune checkpoint inhibition	GU cancer	Phase I	Toxicity	NCT04038619
	Immune checkpoint inhibition	Melanoma	Phase II	Response	NCT04577729
	Allo-HCT	Hematologic cancer	Phase II	Toxicity (GVHD)	NCT03812705
	Allo-HCT	Hematologic cancer	Phase II	Toxicity (infections)	NCT03678493
Microbial ecosystem therapeutics (MET-4)	Immune checkpoint inhibition	Solid tumors	Phase I	Response	NCT03686202
Probiotic (Bifidobacteria)	Immune checkpoint inhibition + chemotherapy	NSCLC	Phase I	Toxicity, surgical complications	NCT04699721
	Chemotherapy	CRC	Phase II	Therapy response	NCT04131803
Probiotic (*Clostridium butyricum*)	Immune checkpoint inhibition	RCC	Phase I	Safety	NCT03829111
	Allo-HCT	Hematologic cancer	Phase I	Toxicity	NCT03922035
Prebiotic (Fiber)	Immune checkpoint inhibition	Melanoma	Phase II	Safety	NCT04645680
	Chemotherapy	Gastrointestinal cancers	Phase II	Toxicity	NCT04447443
	Radiotherapy	Gastrointestinal and GU cancers	Phase III	Toxicity	NCT04534075

Derived from clinical trials registry of the NIH Library of Medicine. allo-HCT, allogeneic hematopoietic cell transfer; CRC, colorectal cancer; GU, genitourinary; RCC, renal cell carcinoma; MET, defined mixture of pure live cultures of intestinal bacteria isolated from a stool sample of a healthy donor; NSCLC, non–small-cell lung cancer.

pathogenesis, but in multiple cases yielded conflicting results. For example, *Lactobacillus reuteri* was shown to competitively co-aggregate with *H. pylori*, with supplemented participants showing a significantly decreased *H. pylori* burden, suggesting that highly targeted bacterial supplements may be effective in cancer prevention (Holz et al., 2015). *Lactobacilli* and *Bifidobacteria* were suggested to reduce tumor incidence, progression, and volume in azoxymethane/dextran sulfate sodium (AOM/DSS) CRC models in mice (Lee et al., 2015; Talero et al., 2015) and 1,2-dimethylhydrazine dihydrochloride (DMH) CRC models in rats (Gamallat et al., 2016) by increasing SCFA production, inducing apoptosis, and inhibiting cancer cell proliferation (Gamallat et al., 2016; Hu et al., 2015). In addition, some probiotics convincingly ameliorated adverse effects of chemo- and radiotherapy, as summarized recently (Rodriguez-Arrastia et al., 2021). Conversely, several studies demonstrated that probiotic supplementation has shown little or no effect, or even induced adverse reactions, upon administration to cancer patients. For example, a clinical study following patients receiving chemotherapy for acute myeloid leukemia demonstrated no significant changes in immunosuppression upon *Lactobacillus acidophilus* or *Saccharomyces boulardii* supplementation. Moreover, this probiotic supplementation actually increased systemic infection rate in supplemented patients (Przybylski and Reeves, 2017). Likewise, *Lactobacillus* spp. supplementation featured little ability to control radiotherapy-induced diarrhea in endometrial adenocarcinoma patients (Giralt et al., 2008), while *Lactococcus brevis* failed to ameliorate oral mucositis in head and neck cancer patients (De Sanctis et al., 2019). Of note, probiotic usage may be associated with significant consequences inflicted upon the indigenous microbiome, including inhibition of microbial reconstitution following antibiotics treatment (Suez et al., 2018), enhancement of the antibiotics resistance landscape (Montassier et al., 2021), and in some cases systemic and localized infections that may be hazardous in the immunosuppressed cancer setting (Kothari et al., 2019).

These disparate and at times conflicting results may be driven by individualized colonization resistance to exogenous probiotics mediated by the indigenous microbiome (Zmora et al., 2018). Developing new ''precision probiotic'' preparations featuring an optimized gut colonization capacity with reproducible cancer responsiveness, while ensuring patient safety, constitutes an exciting field of active research (Veiga et al., 2020). Such efforts integrate a combination of phenotypic screening pipelines, including elucidation of secreted small molecule impacts on tumor and immune cells, *in silico* prediction of new bioactive ''precision probiotics,'' and microbial engineering (Veiga et al., 2020). Such precision probiotic preparations would potentially be harnessed to the individual, based on their microbiome and clinical features, and used as an adjuvant for cancer therapy.

Microbial targeting into tumors

In addition to orally delivered whole microbiome configurations or isolated probiotics, systemically administered tumor-associated microbes have been contemplated for decades to constitute potential tumor-homing vehicles. Utilizing the migration-attraction components of *Salmonella* spp., attenuated mutant

strains were studied in a phase I trial but lacked tumor specificity (Cunningham and Nemunaitis, 2001; Duong et al., 2019). With further modifications, improved tumor specificity and antitumor activity via activation of the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome and induction of IL-1B, IL-18, and TNFα, were noted in mouse models (Kim et al., 2015). Recently, a guanosine tetraphosphate (ppGpp)-attenuated *Salmonella* strain could activate TME innate immune and proinflammatory pathways in mice, leading to an overall disruption in tumor vasculature (Yi et al., 2020).

In addition, transferred commensal members of the gut microbiome can be utilized as synthetic biology chassis (Charbonneau et al., 2020). Bacteria have evolved a wide range of potentially useful physiological and metabolic properties that can be leveraged in penetrating and modulating the TME (Adams, 2016). For instance, *Salmonella typhimurium* mutants engineered to express hemolysin E and altered quorum sensing pathways were able to effectively target tumor sites and lyse to reduce tumor activity and increase survival in a subset of mice (Din et al., 2016). The probiotic *E. coli* Nissle 1917 strain was modified to produce nanobodies that would intratumorally target PD-L1 and CTLA-4, and showed a promising tumor reducing capacity (Gurbatri et al., 2020). A nonpathogenic *E. coli* strain has been engineered to express azurin, a small bacterial protein shown to induce apoptosis in tumor cells and prevent metastasis in mice (Adams, 2016), or to lyse specifically within the TME while releasing an anti-CD47 antagonist nanobody to activate tumor-infiltrating T cells (Chowdhury et al., 2019). *Bifidobacterium infantis* has been reprogrammed to express cytosine deaminase to convert nontoxic 5-fluorocytosine into the cytotoxic compound 5-FU. Administration of the prodrug together with the deaminase-expressing *Bifidobacterium* strain inhibited melanoma growth in mice (Yi et al., 2005). To avoid bacteria-induced and tumor lysis–induced systemic inflammatory immune responses and resultant cytokine release syndromes, kill switches are being programmed in engineered bacteria to control replication in the host and duration of activity while limiting potential toxicities. Rigorous clinical trials are required to evaluate beneficial effects of transferred microbes in the human cancer setting, while ensuring patient safety.

Diet

Dietary modifications constitute an attractive way to shape the microbial population (mainly, but not exclusively in the gastrointestinal tract) toward a 'healthy' configuration, while enhancing the effectiveness of cancer therapies. "Westernized" diets high in fat (Schroeder et al., 2018), refined carbohydrates (Reynolds et al., 2019), and fructose (Kumar et al., 2021) may contribute to carcinogenesis in CRC (Aardema et al., 2021; O'Neill et al., 2016), prostate (Drake et al., 2012), and breast cancers (Kumar et al., 2021). Switching to a high-fat diet increases the prevalence of Firmicutes and Proteobacteria, while decreasing the abundance of Bacteroidetes (Hildebrandt et al., 2009) and activating NF-κB inflammatory pathways in mice, which correlates with cancer onset (Kim et al., 2012). Conversely, a high-fiber diet induces bacterial production of SCFAs including butyrate, propionate, and acetate (Bultman, 2014; Garcia-Mantrana et al., 2018), which feature both anti-inflammatory and pro-apoptotic properties in mouse cancer models (Donohoe et al., 2014; Schroeder et al., 2018). Feeding mice an oral administration of inulin, a polysaccharide dietary fiber, significantly enhanced the efficacy of anti-PD-1 therapies in adenocarcinoma mouse models (Han et al., 2021), while increasing the intestinal abundance of *Akkermansia* and associated fecal SCFAs, previously linked to increased ICI efficacy (Routy et al., 2018b). Inulin administration further led to immunological changes within the TME, characterized by increased PD-1+ CD8+ T cells and reduced regulatory T cells.

However, most of these studies have been carried out in laboratory animals, while disregarding "real-life" interindividual variability in human physiological and disease-related responses. In recent years, the Personalized Nutrition Project (Zeevi et al., 2015) and the PREDICT1 study (Berry et al., 2020) reported that microbiome- and host features from two large human cohorts could be utilized to develop personalized machine learning-based predictions of human postprandial glycemic and lipidemic responses to food. Indeed, personalized prediction-based dietary modifications could attenuate short-term (Zeevi et al., 2015) and long-term (Ben-Yacov et al., 2021) metabolic consequences of post-prandial glycemic spikes in prediabetic individuals. Similarly harnessing human dietary responses to the cancer setting may enable tailoring diets to the individual and their microbiome, in preventing or treating cancer and its complications, while optimizing treatment responsiveness. Such approaches constitute exciting avenues of ongoing research.

Postbiotic therapy

Nondefined mixtures of bioactive microbial compounds, such as exopolysaccharide (EPS) preparations and cell-free supernatants (CFS) from *Lactobacillus* spp., were suggested to be bioactive in some cancers. *In vitro*, EPS induced decreased proliferation of liver and GI tumor cell lines (Wang et al., 2014), while CFS preparations from *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium* spp. induced cellular apoptosis, decreased tumor cell proliferation, and activated anti-inflammatory signaling pathways (Homayouni Rad et al., 2021). Similarly, beta-glucans, a complex group of glucose polymers found in the cell wall of bacteria and yeast, enhanced leukocyte killing of tumor cells through C3b complement coating of tumor cells (Hong et al., 2003).

Refinement of these approaches, by characterization of well-defined bioactive microbial compounds and their supplementation as cancer treatment is termed "postbiotic" therapy. For example, mevalonate and dimethylglycine were enriched in the intestinal contents and sera of germ-free mice colonized with rationally selected Bacteroidales, *Eubacterium,* and *Faecalibacterium* strains and associated with enhanced antitumor immunity and ICI efficacy (Tanoue et al., 2019). Indoles, major bacterial metabolites of tryptophan metabolism and ligands of the aryl hydrocarbon receptor (Gutierrez-Vazquez and Quintana, 2018), are essential for mucosal homeostasis (Alexeev et al., 2018). Indeed, serum levels of 3-indole propionic acid were reduced in patients with ovarian cancers (Ke et al., 2015). Indoles also featured *in vitro* cytostatic activity against breast cancer cell lines and their supplementation induced a reduced metastatic burden in murine breast cancer models (Sari et al., 2020).

An alternative postbiotic approach may harness OMVs as delivery vehicles of tumor-modulating cargo. A modified OMV from

E. coli showed promising results as a cancer immunotherapeutic agent in mouse models of colorectal cancer, by accumulating in tumors and producing IFNγ to increase the antitumor response within the TME (Kim et al., 2017). An antigen-decorated OMV-vaccine elicited a specific antitumor immune response with abrogation of lung melanoma metastasis and inhibition of subcutaneous CRC growth (Cheng et al., 2021). Expanded identification of dietary, host, or microbial cancer-modulating small molecules and OMVs will likely constitute an exciting avenue of cancer-microbiome research in the coming decade.

Antibiotic treatment

Antibiotics-induced microbial eradication in cancer is only indicated in preventing gastric carcinoma and MALT lymphoma by *H. pylori* elimination (Cheung and Leung, 2018; Kamboj et al., 2017; Selgrad and Malfertheiner, 2008). Even in this particular setting, given that *H. pylori* is a commensal which in most cases is not associated with cancer development, universal eradication remains debatable (Kakiuchi et al., 2021; Watanabe et al., 2020). Experimental antibiotics have been recently targeted against intratumoral bacteria that can metabolize gemcitabine as a means of restoring the efficacy of gemcitabine in laboratory mice subcutaneously injected with MC-26 carcinoma cells (Geller et al., 2017). A recent phase III study is assessing this application as a treatment of PDAC (Guenther et al., 2020). However, broad-spectrum antibiotic treatments can also have deleterious effects on tumor progression. In patients treated by allogeneic hematopoietic cell transfer therapy for hematologic malignancies (Shono et al., 2016) and ICIs for advanced melanomas (Elkrief et al., 2019), antibiotics mediated the loss of *Bifidobacterium* spp. or *Akkermansia* spp. and were associated with depletion of microbial metabolites such as SCFA, collectively attenuating treatment efficacy. Likewise, cancer patients treated with broad-spectrum antibiotics up to 30 days before initiation of ICI had a significantly reduced overall survival compared with nontreated patients or patients who received antibiotics at later time points (Pinato et al., 2019). As antibiotics are commonly indicated in cancer patients as life-saving treatments in combating infection, strategies are being developed to minimize antibiotics-mediated impacts on the indigenous microbiome. One such investigative strategy involves colon-targeted antibiotic adsorbents (de Gunzburg et al., 2018), currently evaluated in a phase III study in patients suffering from acute myeloid leukemia or myelodysplastic syndrome. Collectively, antibiotics usage for eradication of cancer-promoting pathobionts will likely remain limited, given their indiscriminate impacts on the indigenous microbiome, which may lead to adverse effects, emergence of resistant strains, and unforeseeable impacts on disease course and treatment efficacy. New modalities enabling targeted elimination of cancer-promoting commensals, while bearing minimal microbiome impacts, constitute an area of active research.

Phage therapy

Bacteriophages, or viruses capable of infecting bacteria, are ubiquitous in nature, and potentially impact the gut microbiome composition though waves of "phage bloom" (Duerkop et al., 2018). Bacteriophages have been promoted as targeted antibacterial treatment for decades (Federici et al., 2021). Systemic administration of individual phages (Chhibber et al., 2008) or cocktails of semi-related phages (Sarker et al., 2012) featured preliminary effectiveness against bacterial infections in mice (Nale et al., 2016; Watanabe et al., 2007) and human patients (Petrovic Fabijan et al., 2020). Challenges in systemic phage treatment include development of bacterial phage resistance, potential immunogenicity, and rapid phage clearance mediated by complement activation and phagocytosis in the liver and spleen (Capparelli et al., 2006; Hodyra-Stefaniak et al., 2019). Oral phage administration may circumvent systemic immunogenicity while targeting disease-associated gut commensals without bearing major collateral effects on other members of the microbiome. Indeed, fecal microbiome transplantation of bacterial-depleted viral-like particles (90% of which were bacteriophages) altered high-fat diet-induced dysbiosis in mice (Lin et al., 2019), and was demonstrated to be feasible in humans, although significant interindividual colonization differences were noted between viral FMT recipients (Draper et al., 2018; Zuo et al., 2018). More well-defined phage cocktail treatments targeting disease-associated pathobionts through multiple receptors may enhance specificity of pathobiont targeting while preventing the emergence of phage resistance (Federici et al., 2021). However, such an approach faces many challenges. Despite the lack of host phage receptors, the innate immune system may sense phage DNA, leading to immune reactivity in some cases (Gogokhia et al., 2019). Moreover, gastrointestinal phage delivery may be complicated by biophysical conditions including gastric acidity, the intestinal mucus layer, and associated biofilm structures, collectively inactivating phages or limiting their engagement to their bacterial targets (Capparelli et al., 2006; Dabrowska and Abedon, 2019) (Wolochow et al., 1966).

In cancer, the use of phage preparations may enable targeted eradication of cancer-promoting commensals, while bearing minimal impacts on the surrounding microbiome. Initial studies have shown promise in eradication of the cancer-associated commensal *H. pylori* by a species-specific lytic bacteriophage in an *in vitro* model (Cuomo et al., 2020). Alternatively, therapeutic cargo-harboring phages could be directed to target cancer-residing bacteria, enabling their cargo release within the TME. For example, *F. nucleatum*–specific phages have been engineered to carry irinotecan nanoparticles. Once the phages migrated to CRC sites populated by *F. nucleatum*, the nanoparticles were released at the TME (Zheng et al., 2019).

CHALLENGES IN MICROBIOME AND CANCER RESEARCH

With enhanced exploration of the microbiome and its contributions to health and disease, accumulation of massive amounts of computational and experimental data holds promise in enhancing our understanding of disease processes, cancer included, while harnessing the knowledge towards development of new diagnostic and therapeutic modalities. However, such multidisciplinary research is faced with significant technological and conceptual hurdles that must be recognized and addressed in order to reach an accurate and actionable understanding of host-microbiome contributions to health and disease.

Sample allocation

Most microbiome studies harness stool or oral microbiome samples as accessible proxies for GI microbiomes. However, such

niche correlations are limited by inherent differences between microbiome configurations in stool, and those along the GI tract (Zmora et al., 2018). Computational inference of gut microbiome community structure from that of stool, or direct, minimally invasive gut microbiome sampling, may optimize the accuracy and reproducibility of cancer-microbiome assessments in years to come. Even more challenging is the prospect of assessing the human tumor microbiome. While difficult to obtain, intratumoral microbial signals may prove to be clinically valuable. For instance, diversity of the tumor microbiome at the time of tumor surgery may predict survival in patients with pancreatic cancer (Riquelme et al., 2019). Obtaining such samples and utilizing them as part of the clinical decision-making repertoire is challenging, but expected to constitute an exciting field of research in coming years.

Data and resource availability

Advances in NGS, metabolomics, and proteomics have enabled high-throughput data generation in identifying specific compositional and functional microbial signatures associated with cancer development or the efficacy of anticancer therapies. One major challenge in this respect relates to data and resource availability, a critical need in optimizing microbiome research in the face of the general scientific "reproducibility crisis" (Knight et al., 2018). An additional challenge relates to insufficient harmonization of data acquisition and analysis methodologies and techniques (e.g., DNA extraction methods or *16S rRNA* gene PCRs), collectively limiting the ability to compare, integrate, and probe datasets from across different studies and geographic regions, while generalizing their results. Encouragingly, publication of microbiome-centered papers increasingly mandates that sequencing data and detailed methods are made publicly available, through services such as the European Nucleotide Archive or the Sequence Read Archive of NIH. However, these data frequently lack sufficiently detailed accompanying metadata, partly due to limitations imposed by local institutional review boards despite patient de-identification. Likewise, sharing of sample size and statistical power calculations (Casals-Pascual et al., 2020) is often not sufficiently implemented. Importantly, web applications are now available for these purposes, for instance https://fedematt.shinyapps.io/shinyMB/ (Mattiello et al., 2016). The next decade of microbiome research will require better harmonization and data sharing, including of bioinformatic analytic tools (Adlung et al., 2021), enabling more uniform denoising, host read removal, and taxonomic and gene database alignments.

Interindividual microbiome variability

In addition to the above described technical variabilities impacting microbiome-generated results, biological inter- and intra-individual variations, stemming from personalized uniqueness of microbiome configurations, constitute a formidable challenge in generalizing results, while differentiating between signal and noise. For instance, three recently published landmark studies investigating gut microbiome profiles as predictors of the efficacy of ICIs revealed different taxa to be associated with the outcomes of immunotherapy (Gopalakrishnan et al., 2018b; Matson et al., 2018; Routy et al., 2018b). Such a considerable variability in microbiome data is often criticized (Schloss, 2018), yet may represent true biological variability that does not necessarily stem from methodological imperfections. Multiple host and environmental factors impact this interindividual variability, including geography, age, gender, lifestyle features (Yatsunenko et al., 2012), genetics (Kurilshikov et al., 2021), and underlying disease (Manor et al., 2020). Importantly, interindividual microbiome variability is not always a caveat, but may enable identification of personalized- and disease context–specific microbiome contributions to differential disease manifestations in face of similar genomic risk factors. Such personalized microbiome "signatures," representing a big data fingerprint, may facilitate machine learning prediction of individualized physiological and cancer-related traits and responses (Zeevi et al., 2015). As an integral component of these efforts, new computational tools will likely be needed to accurately capture precise patterns of person- and disease-specific microbiome kinetics diverging over time and their longitudinal impacts on clinical disease readouts.

Correlation versus causation

As featured throughout this review, one of the most important challenges to the field of cancer-microbiome research is to evolve beyond identification of associations and correlations, toward the establishment of causality and mechanism. A widely used modality in demonstrating microbiome causation involves the transfer of whole microbiome configurations, defined consortia, or single microbes into germ-free mice, allowing researchers to model the impact of microbes on human cancer and therapy. Using these methods, germ-free mice administered the pro-carcinogenic agent azoxymethane (AOM) and colonized with fecal microbiomes from patients with CRC featured higher proportions of Ki67-positive proliferating cells and inflammatory markers, as compared to germ-free mice colonized with fecal microbiomes from healthy controls. Moreover, human fecal microbiome transfer from CRC patients into conventional mice enhanced macroscopic polyps and high-grade intestinal dysplasia in recipients, as compared to controls (Wong et al., 2017). Likewise, experimental mono-colonization with enterotoxigenic *B. fragilis* or *E. coli* containing *pks* genotoxic islands (necessary for colibactin synthesis) isolated from biofilms on human colonic polyps accelerated intestinal tumor development in the AOM and $APC^{Min/+}$ tumor mouse models (Dejea et al., 2018).

In addition to the *in vivo* mouse setting, *in vitro* models also provide important means of testing direct or indirect impacts exerted by microbes and their secreted molecules on cancer. For instance, microfluidic gut-on-chip models were developed to co-culture human gut epithelial cells and commensal microbes under anaerobic conditions to study inflammatory processes or host-pathogen interactions (Grassart et al., 2019; Shah et al., 2016). Three-dimensional organoid models generated from small or large intestines enable to decode mechanistic impacts of distinct commensals and their bioactive products on cancer development and progression. For example, *F. nucleatum* co-cultured with human tumor organoids induced the gene expression of pathways involved in cancer metastasis (Kasper et al., 2020). Human intestinal organoids co-cultured with a colibactin-secreting *E. coli* strain developed a distinct

mutational signature with single-base substitutions and gene mutations characteristic of human CRC (Pleguezuelos-Manzano et al., 2020), thereby unraveling genotoxic contributions of cancer-promoting commensals. New methodologic advances enable the integration of immune and other TME cells and complex microbial communities into the organoid setting. Indeed, patient-derived organotypic tumor spheroids that retain immune cells may even be capable of responding to immune checkpoint inhibition in short-term 3D cultures (Jenkins et al., 2018).

CONCLUDING REMARKS

In less than a decade, the young microbiome field has provided valuable insights into commensal contributions to human physiology and disease. Human cancer, representing one of the most complex, devastating, and poorly understood human pathologies, has been linked to distinct microbial changes and global alterations in microbiome community structure. Investigating causal and molecular interactions between commensal microbes in mucosal body sites and in the TME is expected to shed new light on human variability in cancer development, progression, and treatment responsiveness. Such research is faced with formidable challenges related to sample allocation, processing, sequencing, and data analysis, in addition to striving to evolve from a correlative to causative understanding of microbial influences on cancer. With these challenges notwithstanding, microbial contributions to cancer biology will likely take center stage in the next decade of cancer research, while increasingly contributing to cancer diagnosis, patient stratification, and treatment.

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All authors performed an extensive literature research, contributed substantially to discussion of the content, wrote, and edited the manuscript. N.C. and C.A.A. equally contributed to this work.

DECLARATION OF INTERESTS

R.S. is a salaried scientific consultant for Micronoma, BiomX, Biomica, and CuResponse. E.E. is the scientific founder of DayTwo and BiomX, and a salaried scientific consultant to Roots Health GmbH in topics unrelated to this work.

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