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Hot Topic

Bugs as drugs: The role of microbiome in cancer focusing on immunotherapeutics

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ABSTRACT

The human microbiome comprising microorganisms, their collective genomes and metabolic products has gained tremendous research interest in oncology, as multiple cohorts and case studies have demonstrated discernible interpatient differences in this ecosystem based on clinical variables including disease type, stage, diet, antibiotic usage, cancer treatments, therapeutic responses and toxicities. The modulation of the gut microbiome is the subject of many ongoing preclinical and clinical investigations, through the manipulation of diet, as well as the use of prebiotics, probiotics, specific antibiotics, fecal microbial transplantation, microbial consortia and stool substitutes. Standardization and quality control are needed to maximize the information being generated in this growing field, ranging from technical assays to measure microbiome composition, to methodological aspects in the analysis and reporting of results. Proof-of-mechanism and proof-of-concept clinical trials with appropriate controls are needed to confirm or refute the feasibility, safety and ultimately the clinical utility of human microbiome modulation in cancer patients.

Introduction

The microorganisms living on and in the human body including bacteria, viruses, fungi, protozoa and other microbes, as well as their collective genomes and metabolic products constitute the human microbiome. The field of microbiome research in cancer has grown substantially over the past decade, with a nearly 46-fold increase in publications from 2009 to 2019 (37 to 1727articles) based on PubMed using the search terms “microbiome” and “cancer”. The bacterial component of the intestinal microbiome has gained specific interest in oncology as accumulating evidence indicates a strong association with host anticancer immune responses, influencing the efficacy and toxicity of immune-checkpoint inhibitors (ICI) [1–6]. There is a complex relationship between the gut microbiome, the immune system and metabolic homeostasis [7]. Gut commensal bacteria and their metabolites are essential for the development and maturation of the host immune system starting from early stages of life [8]. They are responsible for regulating both innate and adaptive immune responses not only locally at the level of the intestinal mucosa but also systemically [8]. Pathology-associated alterations in microbiome composition, so-called dysbiosis, can precipitate disruption of mucosal barriers, cytokine-release, impaired antigen priming, myelopoietic dysregulation, and imbalance of immune cell subsets, ultimately leading to increased susceptibility to infections, immune-related disorders and cancer [9–11].

Microbiome, cancer and host immunity

Infections by non-commensal bacteria and viruses are well established contributors to the tumorigenesis of solid tumors, such as Epstein-Barr virus in nasopharyngeal cancer, human papillomavirus (HPV) in cervical and oropharyngeal carcinomas, and *Helicobacter pylori* in gastric adenocarcinoma [12–14]. Beyond the existing cause-effect relationship between specific tumor types and the implicated pathogenic microorganisms, changes in the total quantity and relative abundance of commensal bacteria can also trigger cancer development and progression. Several studies have shown different gut microbiome composition among patients with colorectal and pancreatic malignancies compared to healthy controls [15–17]. Similarly, increased relative abundance of

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Corynebacterium and *Kingella* bacteria in the mouth (oral microbiome) has been associated with reduced incidence of oral cavity carcinomas [18]. Although the underlying mechanisms are not fully understood, changes in microbiome composition are thought to induce or exacerbate chronic inflammation, which may disrupt immune surveillance ultimately affecting local and distant carcinogenesis [19,20]. Furthermore, multiple external factors such as diet, antibiotics, infections and smoking, as well as host-dependent intrinsic characteristics such as genetic susceptibility, can alter the composition of the microbiome and potentially amplify or mitigate the risk of cancer [21–24].

While the intestinal microbiome has been the most extensively evaluated, bacterial communities from other body compartments such as oral, genitourinary and respiratory microbiomes as well as tumor-associated microbiomes also play a relevant role in local tumorigenesis and the risk of metastasis [25,26]. Multiple studies have shown a correlation between a particular tumor and its surrounding microbiome (e.g. oral microbiome and oral cavity cancers), suggesting local microbiome signatures may be used as tumor-specific diagnostic biomarkers [25,27]. For instance, the composition of the oral microbiome measured in saliva and/or tumor of patients with head and neck carcinomas differs

Table 1
Selected studies evaluating the role of microbiome in modulating response to immunotherapy.

Author	Year	Mice vs. Human	ICI	Type of sequencing	Tumor Type	Outcome	Finding
Sivan et al. [4]	2015	Mice	Anti-PD1	16S rRNA sequencing	Melanoma	Response	The presence of *Bifidobacterium* was correlated with antitumor T-cell responses and improved anti-PD1 efficacy. Transferring fecal material from responders to non-responders appeared to restore responses.
Vetizou et al. [51]	2015	Mice/ Human	Anti-CTLA4	qPCR	Human: Melanoma / Mice: MCA205 sarcoma, RET melanoma and MC38 colon	Response	*Bacteroides* administration was able to restore anti-CTLA4 responsiveness in germ-free and antibiotic-treated mice. Fecal transplants from patients treated with anti-CTLA4 who harbored Bacteroidales species boosted anti-CTLA4 responses in mice.
Chaput et al. [3]	2017	Human	Anti-CTLA4	16S rRNA sequencing	Melanoma	PFS, OS	Patients whose baseline microbiome was enriched with *Faecalibacterium* genus and other Firmicutes (unclassified *Ruminococcaceae*, *Clostridium* XIVa and *Blautia*) had longer PFS, OS and a higher incidence of colitis, when compared to patients with baseline microbiome driven by Bacterioides.
Frankel et al. [48]	2017	Human	Anti-CTLA4, Anti-PD1, Combination of Anti-CTLA4 + Anti-PD1	Metagenomic shotgun sequencing; Metabolomics	Melanoma	ORR	Patients with baseline microbiome enriched for *Bacteroides caccae* and *Streptococcus parasanguinis* had better ORR. Metabolomics revealed high levels of anacardic acid in responders.
Gopalakrishnan et al. [6]	2018	Human/ Mice	Anti-PD1	16S sRNA and Metagenomic shotgun sequencing in a subset	Melanoma	ORR, PFS	Responders had higher alpha diversity and higher relative abundance of Ruminoccaceae bacteria. Shotgun sequencing identified *Facalibacterium* genus as enriched in responders. Those patients were associated with increased PFS. Germ-free mice receiving fecal transplants from responding patients were able to restore antitumor immunity.
Matson et al. [5]	2018	Human/ Mice	Anti-PD1	16S rRNA and Metagenomic shotgun sequencing	Melanoma	ORR	Responders were associated with higher abundance of *Bifidobacterium longum*, *Collinsella aerofaciens* and *Enterococcus faecium* at baseline. Germ-free mice receiving fecal transplants from responding patients were able to restore antitumor immunity.
Routy et al. [1]	2018	Human/ Mice	Anti-PD1	Metagenomic shotgun sequencing	Human: NSCLC and RCC / Mice: MCA-205 sarcoma and RET melanoma	ORR, PFS	Baseline samples of responders were enriched for *Akkermansia muciniphila* and classified and unclassified Firmicutes. Germ-free mice receiving fecal transplants from responding patients were able to restore antitumor immunity. Administration of *Akkermansia muciniphila* was able to restore antitumor immunity in germ-free mice receiving fecal transplants from non-responders.
Peters et al. [49]	2019	Human	Anti-CTLA4, Anti-PD1, Combination of Anti-CTLA4 + Anti-PD1	16 sRNA and Metagenomic shotgun sequencing	Melanoma	PFS	Higher microbial diversity was associated with longer PFS. Patients enriched for *Faecalibacterium prausnitzii, Streptococcus sanguinis* and other protective species were associated with longer PFS, whereas patients enriched for Bacteroides had shorter PFS.
Wind et al. [50]	2020	Human	Anti-PD1, Combination of Anti-CTLA4 + Anti-PD1	Metagenomic shotgun sequencing	Melanoma	OS, PFS	No difference in alpha-diversity between responders and non-responders. Carriers of *Streptococcus parasanguinis* had longer OS. Patients enriched for *Peptostreptococcaceae* (unclassified species) were associated with shorter OS and PFS.

from that in healthy individuals, and also varies based on tumor location, tumor volume, HPV status and treatment received [28]. Similar findings have been reported regarding the vaginal microbiome in patients with HPV-related cervical carcinomas, or the urine microbiome in patients with prostate and bladder cancers [29–32]. The relative abundance of *Fusobacterium nucleatum* in tumor tissue of patients with colorectal and esophageal cancers was found to be an independent predictor of disease-free survival, and was associated with resistance to platinum-based chemotherapy [33,34]. In murine colorectal cancer models, *F. nucleatum* is able to suppress local antitumor immune responses by upregulating NFκB pathway and increasing the expression of inhibitory immune checkpoints in T cells, which may explain the detrimental effect in survival [35–37]. Patients with pancreatic cancer who have increased intratumoral Gammaproteobacteria were found to be resistant to gemcitabine, by metabolizing this agent into inactive products [38]. Furthermore, Nejman et al. recently studied the tumor microbiome of seven different solid cancer tumor types, and found that its composition is tumor-specific and impacts treatment response [39].

Beyond the tumor-associated microbiome, gut commensal bacteria can also enhance or reduce the efficacy and toxicity of cancer treatments including chemotherapy, radiotherapy and immunotherapy [5,40,41]. One of the many functions of the gut microbiome is to metabolize nutrients and drugs, including some chemotherapy agents [42]. Preclinical and clinical studies have indicated that selective alteration of gut composition using antibiotics can impair responses to platinum and cyclophosphamide chemotherapies in several cancers [43,44]. In other cases, tumor sensitivity to specific chemotherapies such as oxaliplatin or cyclophosphamide also depends on gut microbiome-mediated local and systemic immune responses [33,45,46]. Beyond chemotherapy, toxicity from local treatments such as radiotherapy can also be modulated by the microbiome. An altered gut microbiota is associated with early and late radiation enteropathy, while oral dysbiosis can increase the severity of radiation-induced mucositis in patients [41,47].

Gut microbiome modulating response to immune checkpoint blockade

Multiple lines of evidence have suggested a role for the gut microbiome in modulating response to immune checkpoint blockade across several cancer types (Table 1) [1,5,6,48–50]. Many of these studies describe the presence of distinct, favorable gut microbial "signatures" associated with enhanced intratumoral immune infiltrates in patients who have responded to ICI, a prime example being in those with metastatic malignant melanoma on anti-programmed death protein (anti-PD1) therapy [5,6]. Higher gut microbial alpha (within-sample) diversity was noted in responders to anti-PD1 antibody, as was the relative abundance of the order Clostridiales, the *Ruminococcaceae* family, and the species *Faecalibacterium prauznitzii* [6]. In contrast, the microbiome of non-responders demonstrated a lower alpha diversity and higher relative abundance of the order Bacteroidales. Analysis of both the composition of the gut microbiome and the immunological profiling of the tumor microenvironment (TME) demonstrated that the expression of cytotoxic T cell markers and antigen processing and presentation were augmented in patients with favorable gut microbiome [6]. Another study by Matson et al. describes microbiota with higher relative abundance of *Bifidobacterium longum*, *Collinsella aerofaciens* and *Enterococcus faecium* in responders to PD1 blockade [5]. Other studies in melanoma patients whose baseline microbiota was enriched with *Faecalibacterium* genus and other *Firmicutes* showed a longer progression free survival (PFS) and overall survival (OS) upon ipilimumab (anti-cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA4)) treatment than those whose baseline microbiota was enriched with bacteria of the Bacteroidales order [3]. The same pattern has also been described for epithelial tumors treated with anti-PD1 antibodies. For instance, Routy et al. demonstrated that patients with non-small cell lung cancer and renal cell carcinoma who responded to anti-PD1 antibody had their baseline stool samples enriched for *Akkermansia muciphila* and classified and unclassified *Firmicutes* [1].

In addition to human cohort studies, immune response can be modulated in preclinical models through exogenous microbiota transfer to mice. Fecal transplants from patients who responded to anti-PD1 antibody to germ-free mice was able to restore antitumor immunity, whereas the same was not observed with transplant of fecal material from non-responders. Furthermore, administration of *Akkermansia muciniphila* to mice transplanted with non-responders' fecal material was capable of restoring anti-immunity to ICI treatment, suggesting that there are key species capable of driving immunity [1]. Importantly, as of now, there are no established gut microbiome "signatures" universally capable of predicting ICI responsiveness. The association of bacterial taxa with response appears to be context dependent, varying by patient population, experimental design and bacterial species/strain. For example, while Vetizou et al. observed that *Bacterioides fragilis* administration was able to restore anti-CTLA4 responsiveness in germ-free and antibiotic mice [51], Chaput et al. observed that in patients with metastatic melanoma treated with anti-CTLA4 antibody, individuals with baseline gut microbiota composition enriched for *Bacteroides* had shorter PFS and OS compared to those enriched for Firmicutes [3]. While there is convergence amongst findings from published studies, e.g. the association between *Bifidobacterium* species and better responses to ICI [4,5], several key questions as outlined above remain unanswered. Further work to standardize experimental procedures and data interpretation, which are highly variable amongst the studies, are warranted. Transparent sharing of metadata and methodologies are crucial to accurately assess collective findings and put results in perspective. The International Cancer Microbiome Consortium in a recently released consensus outlines this important topic [52].

The underlying mechanisms explaining the correlation between gut microbiome composition and enhanced ICI efficacy are not yet fully understood. Fessler et al. reviewed this topic recently [46]. In their view, identifying the "messenger" that translates a specific signal from the gut into an immune-mediated antitumor response (positive or negative) is key to establish the nature of the microbiome-tumor immunity interactions and to develop therapeutic strategies. These messengers can be either microbiome-dependent (specific bacterial strains; microbe-associated molecular patterns (MAMPS)/pathogen-associated molecular patterns (PAMPS); metabolites), or host-dependent (immune cells and cytokines). For example, live bacteria or MAMPS/PAMPS can act as antigens capable of triggering T cell mediated antitumor responses due to cross-reactivity with tumor antigens (e.g. shared T cell epitopes with *Bacteroides fragilis*), or can act as adjuvants of T cell priming via activation of antigen-presentation cells when translocated into the systemic circulation *(Bifidobacterium sp; Faecalibacterium* genus*)* [4,6,51]. Metabolites produced by certain bacteria (e.g. *Akkermansia muciniphila)* such as short-chain fatty acids can modulate host cytokine production and T cell differentiation, thus enhancing or suppressing antitumor immune responses and efficacy of ICI in preclinical studies [1,53,54]. Host immune cells, and gut dendritic cells in particular, also play a relevant role as messengers of antitumor immunity [43]. Dendritic cells are responsible for immune tolerance to commensal bacteria, and also for T cell priming, differentiation, and activation in response to specific strains or to local mucosal inflammation/damage produced by chemotherapy or anti-CTLA4 agents [45,55–57]. Tanoue et al. revealed that a consortium of 11 different bacterial strains, usually present in low abundance in the human gut microbiome, were able to induce interferon-γ-producing CD8 T cells in the gut via dendritic cell activation, and can thereby increase anti-PD1 antibody efficacy in mouse models [58]. Interferon-γ-producing CD8 T cells were not restricted to the gut, but were found in different organs, suggesting a systemic effect. However, the CD8 T cells were phenotypically distinct from one organ to another, suggesting that bacterial dissemination or merely circulation of gut-origin interferon-γ-producing CD8 T cells were not solely responsible for the observed systemic dissemination. An appealing hypothesis

outlined by the authors is that circulating metabolites produced by the 11-bacterial strains are responsible for the interferon-γ-producing CD8 T cells stimulation. Analysis of cecal metabolomic content of mice colonized with the 11-bacterial strains revealed significant differences in the metabolomic profile when compared to the metabolomic profile of mice colonized with other bacterial strains. This theory is further appreciated by a recent report by Mager et al., demonstrating that the bacterial metabolites inosine and hypoxanthine enhance ICI therapy by the stimulation of a dendritic cell-dependent effector T cell circuit. Inosine, produced by *Bifidobacterium pseudolongum* and *Akkermansia muciniphila* in a context-dependent manner, including the presence of interferon-γ, binds to adenosine 2A receptors eliciting CD8 T cell Th1 differentiation [59], ultimately leading to a stronger immune response.

However, the interactions between gut commensal bacteria and host immunity are complex and can be influenced by a wide variety of host-dependent intrinsic and extrinsic factors (genetic susceptibility, diet, drug use) as well as tumor-specific characteristics such as genomic features, antigenicity, and microenvironment, which can also contribute to the efficacy of ICI agents [60–62].

The effects of antibiotics on response to immune checkpoint inhibitors

The effects of antibiotic treatment on the gut microbiome of mice and consequently impairing anticancer responses and immunotherapy efficacy, have been raised as a possible concern regarding their effects in humans [4,51]. This finding prompted significant interest in investigating the impact of antibiotic treatment in cancer patients, particularly ICI. Several studies have shown a detrimental association between antibiotic use before ICI initiation and worse PFS and OS [61,63–65]. Wilson et al, conducted a metanalysis of observational studies including mainly patients treated with anti-PD1 or anti-PD-L1 agents and concluded that the exposure to antibiotics either prior or during ICI treatment was associated with an inferior OS (HR = 1.92, 95%CI 1.37–2.68) [66]. The effects observed appears to be driven by antibiotic exposure within 42 days of ICI administration (HR = 3.43, 95%CI 2.29–5.14), suggesting a greater detrimental impact of antibiotics in the period immediately prior to ICI administration [66]. This is in line with recent data suggesting that after antibiotic treatment, healthy subjects take between 4 and 6 weeks to re-establish their original microbiome composition [67,68]. The primary limitations with this type of analysis include bias by indication and confounders in those who received antibiotics versus controls. Patients treated with antibiotics are typically in a worse health, with a poorer performance status, and possibly with a higher disease burden. These factors are known to adversely influence the efficacy of ICI agents and should be considered in the interpretation of these findings. Furthermore, antibiotics modulate the gut microbiome in class- or agent-specific ways which may affect their impact on host physiology and ICI responsiveness. These caveats notwithstanding, emerging data suggest that greater detrimental effects are observed in treatment with broad versus narrow spectrum antibiotics, and intravenous versus oral antibiotics [63,69]. Likewise, cumulative antibiotic usage due to multiple or prolonged course appears to be associated with poor clinical outcome [70].

Studies are needed to better elucidate the timing, duration, drug, and host-specific impact of antibiotic use in the outcomes of patients treated with ICI. Although current studies are not sufficiently conclusive to support a recommendation to delay ICI treatment after antibiotic treatment, or to prevent patients from receiving antibiotics when there is a clear indication, physicians should be mindful of the potential detrimental effect of antibiotic exposure prior to ICI initiation and avoid unnecessary use and limit duration whenever it is safe to do so.

Microbiome composition and toxicity to cancer therapy

In addition to facilitating and potentially augmenting therapeutic responses, the gut microbiome has also been associated with cancer therapy toxicity. For example, in the setting of allogeneic stem cell transplantation for hematologic malignancies, compositional differences in the gut microbiota have been associated with varying rates of development of graft-versus-host disease, infection and even mortality [71–73]. In terms of ICI treatment, Chaput et al. demonstrated that patients with metastatic melanoma whose microbiome was enriched with Firmicutes had a higher incidence of colitis after treatment with ipilimumab, as opposed to patients enriched with Bacteroidetes, who had a lower risk of developing colitis [3]. Another study by Dubin et al., involving patients with metastatic melanoma reported similar findings; that a baseline microbiome enriched with Bacteroidetes was associated with a smaller risk of developing ipilimumab-induced colitis [74]. It is unknown whether the same mechanisms cause microbiome-associated treatment response and toxicity, although several unifying mechanisms are possible [75–76]. Specific groups of microbes have been causally linked to promotion of reactive and regulatory immune cells, largely through innate immune recognition and microbial metabolites, respectively [77–78]. Such "non-specific" mechanisms may explain increased immunity and therefore lead to both response and toxicity. For instance, in the aforementioned ipilimumab-induced colitis associated with Firmicutes, a higher frequency of treatment response is also observed, suggesting that the same taxa may be implicated in both phenomena [3]. Conversely, multiple different mechanisms may be at play, some of which are specific to either response or toxicity. For example, cross-reactivity between microbial and tumor or self-antigens has recently been described [79,80]. This phenomenon would be expected to be associated with specific antitumor response or autoimmune toxicity, but without overlap of the two, unless the same antigens are expressed on both tumor and normal cells. Further work is required to elucidate the relationship (and potential overlap) between microbiome composition, ICI efficacy and toxicity development. The determinants of responsiveness and development of toxicity after ICI treatment are likely multifactorial due to interactions between patient, tumor, and molecular factors [81].

Manipulating the gut microbiome may also play a role in treating ICI-induced colitis refractory to standard immunosuppressive treatments. Wang et al. recently described two patients treated with fecal microbiota transplantation (FMT) from a healthy donor in the context of refractory colitis [85]. Both had symptoms ameliorated after the procedure with the presence of donor's bacteria demonstrated on follow-up stool collections. Whether the gut microbiome may predict colitis and other ICI-related toxicities, and if its manipulation plays a role in the management of ICI-related toxicity remains, at this present time, investigational (e.g. NCT04107168, NCT03819296, NCT04107311, NCT04163289).

Measuring microbiome composition

Technical approaches to studying human-associated microbial communities

The evaluation of the microbiome in oncology for clinical research or in practice requires a set of standardized assays, analytical methodology and reporting formats. Human-associated microbial communities can be assayed using culture-based or culture-independent techniques to characterize their composition and function. Three main approaches to do this are: 1) high-throughput amplicon sequencing of genes that function as microbial "barcodes" – the 16S rRNA gene in bacteria and internal transcribed spacer or 18S rRNA genes regions for fungi [86,87]; 2) metagenomic sequencing – bulk sequencing of all nucleic acid in a sample (generally DNA or DNA and RNA) [88] and; 3) analysis of microbial activity including metatranscriptomics, proteomics and metabolomics [89]. Comprehensive culturomics is also employed but has not been as widely applied due to real or perceived practical barriers to implementation [90,91]. Quantitation through qPCR or with other nucleic acid-based detection techniques complement these methods and

provide targeted absolute quantities that can be combined with compositional relative abundance data or used to verify sequencing or culture-based approaches [92].

While standardization of sample handling, sequencing and analytical techniques in microbiome analysis are a focus of significant international collaborative effort, most notably the International Human Microbiome Standards (IHMS) Project (www.human-microbiome.org), these have not been applied systematically to microbiome studies in the field of human cancer research and may not address the unique scientific, logistical and patient-population specific needs of this work. Despite developing or recommending standards is outside of the scope of this review, we do recommend adherence as often as possible to these standards to increase the comparability of results in ICI-microbiome analysis.

Data analysis

Amplicon sequencing characterizes "who is there" by measuring community taxonomic composition, reported as the relative abundance of community members (usually at the level of the genus); metagenomic sequencing provides compositional data at higher taxonomic resolution (even at the strain level), in addition to functional potential – "what they can do" – generally reported as gene complement annotated by functional category [89,93]. Finally, functional profiling defines what microbes are "doing" in an environment at a moment in time by quantifying gene expression or the relative abundance of microbial proteins or metabolites [89,93].

The information generated with these assays is broadly applied in the same way that other biomarkers are and are interpreted based on the study design. Observational studies have characterized microbial

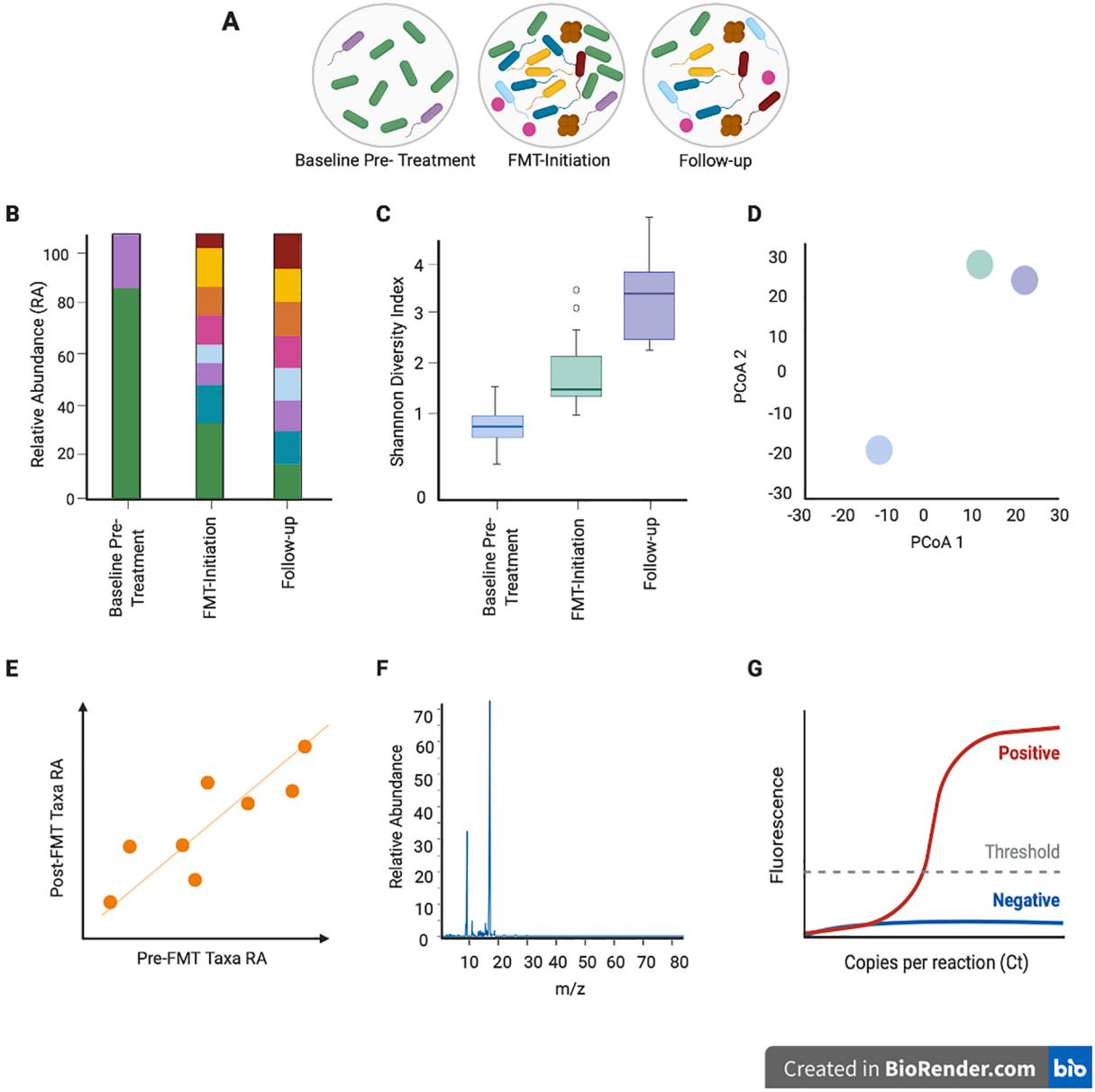

Fig. 1. Measuring microbiome manipulations. A) Hypothetical representation of microbial diversity found in fecal samples at baseline, post-FMT, and at follow-up. B) Relative abundance (RA) of different microorganisms present in panel A. C) Shannon alpha-Diversity index (SDI) (within-sample) showing the follow-up sample with the greatest diversity and richness. D) PCoA plot depicting beta-diversity (between-samples) differences. E) Correlation between Pre and Post RA taxa correlations as a predictor of engraftment in gut microbiome modulator studies using FMTs, probiotics, microbial consortia. F) Mass spectrometry can be used to assess functional capacity of microbial communities as well as TME. G) Total bacterial load and pathogen directed qPCR can be used to confirm 16S rRNA findings from microbiome data. Figure generated in BioRender.

communities at primary tumor sites, in metastases, in non-tumor sites (such as the gut or stool or in tumor-adjacent normal tissue), compared them across tumor, host or treatment characteristics and longitudinally during treatment or disease progression or regression [1,5,6,38,39,45,71,94]). Analysis, based on study design, may be exploratory or hypothesis-testing and while analytical methods and hypotheses vary by study, several common approaches are used, adapted from macroecology and briefly summarized here [95,96] (Fig. 1: 1) Comparisons of alpha-diversity – the diversity within a community – between groups, including number of members (richness), their relative abundances (evenness) and degree of relatedness (phylogenetic diversity); 2) Assessing beta-diversity – the amount of similarity or dissimilarity between two communities, and; 3) Taxonomic composition – the relative (or absolute) abundance of community members.

These approaches and their associated statistical tools can be used to characterize microbes or transcripts, be correlated with host or other parameters, compared between study groups, or analyzed within individuals over time. More recently, they have also been used to assess the impact of microbiome-targeted interventions on microbiome composition and function, including in interventional studies of probiotics, microbial consortia or FMT [97,98]. However, in spite of the increasing incorporation of microbial community assays into clinical studies, there are remaining challenges.

Challenges

As studies evolve from hypothesis generating to hypothesis testing through to validation and clinical translation, we must address multiple ongoing challenges [99–101]. Firstly, technical standards for assays and data pipelining need to be adopted for clinical translational work. Secondly, confirmatory studies must be designed and executed to ensure that the findings from exploratory and hypothesis-generating work are both reproducible and generalizable. Thirdly, reproducible microbe-host-treatment associations must be related to clinically meaningful outcomes in prospective diagnostic or prognostic studies. Finally, treatments designed to augment the microbiome during cancer chemotherapy or immunotherapy must be tested in preclinical models and interventional trials. In microbiome-targeting therapies, basic questions remain to be addressed, including core concepts such as defining potency and the microbial pharmacokinetics: where exogenous microbes reside, whether they survive, proliferate or die, how long they last in a given niche and whether they persist after cessation of therapy, and if so for how long.

Ecological responses – measuring colonization, engraftment and indirect effects

Ecological response to microbiome modulation using FMT, probiotics, and microbial consortia can be assessed using a number of approaches [102]. The most widely used measures include taxonomic composition, alpha- and beta-diversity [102]. Taxonomic impact of therapy can be quantified as the increase or post-treatment absolute or relative abundance of donor taxa in recipient stool post transplantation [102,103]. Engraftment is shaped by a number of variables including resident gut microbiome of recipient, pharmacokinetics and pharmacodynamics of microbiome modulating agent, genetic and phenotypic diversity of donor/microbiome modulating agent and host immune/genetic factors [104]. While transient detection of donor taxa in recipient stool is suggestive of engraftment, colonization entails long term establishment of donor taxa in recipient stool. Taxonomic impact can also be measured by assessing changes in the abundance of endogenous microbes in response to therapy. Effects on global composition may be assessed by comparing alpha-diversity between baseline and post-treatment samples or between intervention and control groups, quantified as species richness/evenness and their composite measures. Beta-diversity (inter-sample compositional differences) can be used to determine distance/dissimilarity between donor microbiota and recipient, intervention groups or pre/post intervention paired samples within an individual. This measure can help investigators understand how distant or similar the donor and recipient microbiota are. Fig. 1 exemplifies how microbiome manipulations can be measured.

Clinical endpoints

The overarching goal of any therapeutic intended to treat metastatic cancer patients is to either increase their OS and/or to improve their quality of life (QOL). As of now, microbiome-targeting therapies are still in an early stage of investigation, and there is no proven correlation between ecological changes in microbiome induced by microbiome-targeting therapies and OS or QOL improvement. The evaluation of the clinical impact of microbiome-targeting therapeutics should be pursued using measures and approaches employed for evaluation of other novel cancer therapies at early stages. For instance, trials demonstrating signals of efficacy, or an improvement in ICI-related toxicity profile are desired. Furthermore, longitudinal collection of data from interventional trials will be crucial for shedding light in mechanistic aspects of microbiome-modulation and interactions with the immune system, as well as potentially identifying correlations between microbiome-changes and relevant clinical endpoints.

Lim itations

The primary limitations of existing studies implicating the microbiome in cancer pathogenesis and therapeutic responsiveness are the same as those for other human observational studies, including a lack of clear understanding of causality, confounders, and the specific limitations of each study design [100,105]. Preclinical mouse models can help establish a mechanistic understanding of the effects of manipulating the microbiome, but they are limited by biological differences between mice and humans, including those specific to their microbiomes [105]. Humans and mice are colonized by different microorganisms, have different immune responses and differing diets/environments which can affect both microbiome composition and microbe-host-tumor-treatment interactions. There are a number of confounders to microbiome studies including co-morbidities, genetics, age, sex, diet, systemic therapy exposure and environment [100]. While combined human observational and preclinical studies have established strong associations and causal relationships between the microbiome, cancer and anti-cancer therapy, large prospective cohorts with defined hypotheses and multi-centered double-blind randomized controlled trials are needed to specifically address the diagnostic, prognostic and therapeutic significance of the microbiome in human cancers.

Manipulating the gut microbiome

Advances in our understanding of the intricate microbial network present in the gut and its impact on the immune response in cancer patients has resulted in the development of novel therapeutic strategies to manipulate the microbiome, or its metabolic function, in an attempt to augment response to ICI and minimize treatment related adverse events. Some of the main strategies to manipulate the gut microbiome are summarized in Fig. 2.

Diet

The role of diet as a key determinant in the composition of the gut microbiota, impacting nutrient extraction and mediating many of the dietary benefits within the human body, is well established [106,107]. The Mediterranean diet, composed of high fiber and low red meat intake, is associated with higher microbiome diversity, as compared to a western diet, composed of high animal fat and protein with low microbiome diversity [108,109]. More specifically, a protein-based diet is associated with increased counts of *Bacteroides* and *Clostridia*, and decreased counts of *Bifidobacterium* compared to a plant-based diet

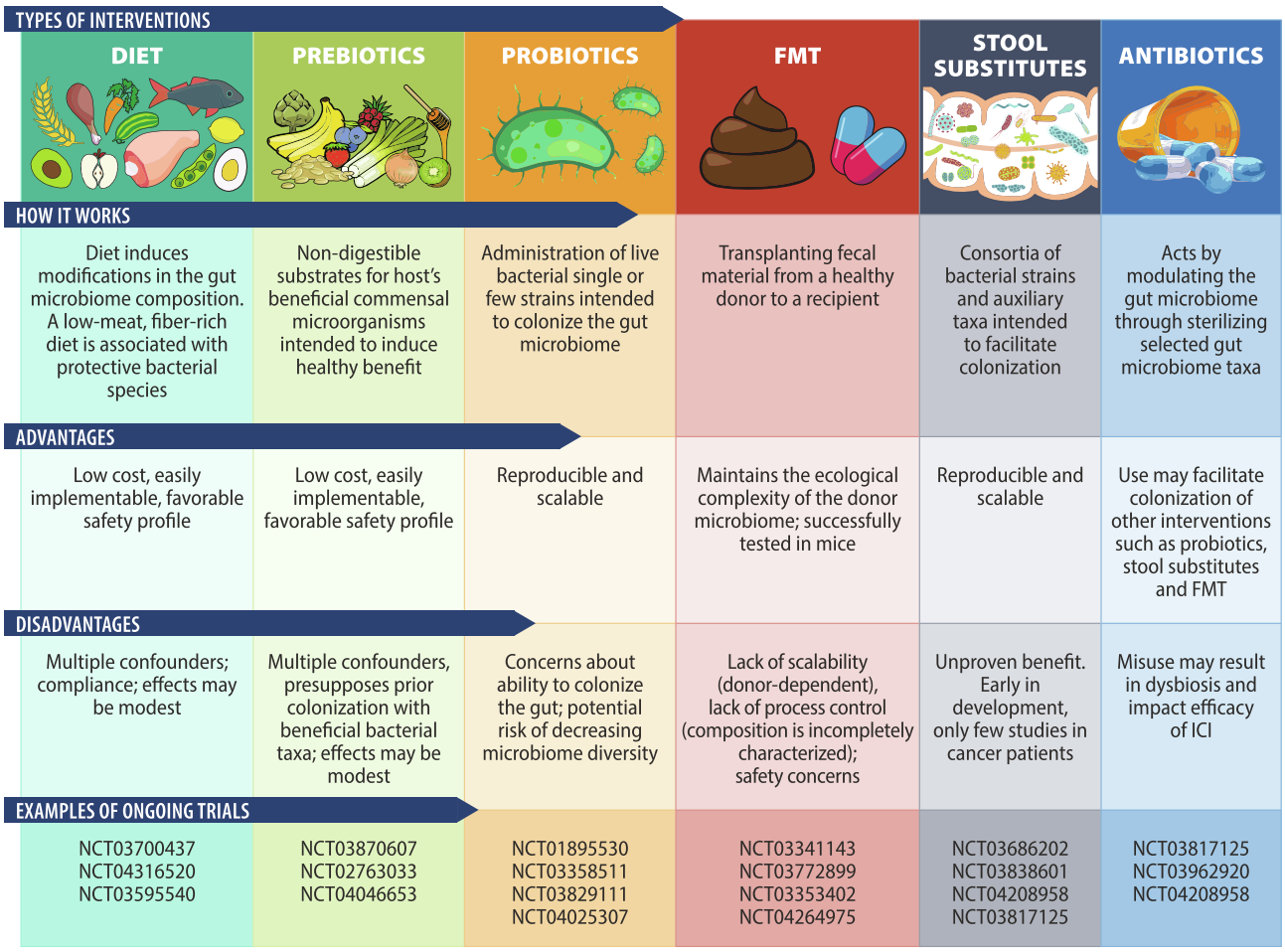

Fig. 2. Summary of the modalities of intervention for modifying the gut microbiome.

[110]. Dietary modifications are capable of altering the gut microbiome composition as early as 24 h after commencing a new diet and takes approximately 48 h to revert back after diet discontinuation [110]. While these modifications have been shown to influence immune responses in mice [111], the role of diet in specifically enhancing ICI-responses in cancer patients, however, is investigational. Many trials investigating the role of dietary modification and fiber supplementation as adjuvants to ICI treatment are currently active (NCT03700437, NCT04316520, NCT03595540). Importantly, the aforementioned dietary strategies (Mediterranean and plant-based) work by promoting metabolic changes induced by some degree of starvation, eventually leading to microbiome modifications. The role of adding or removing a single nutrient class (e.g. carbohydrates, proteins or lipids) from diets, and the potential changes in the microbiome composition and immunomodulation that this may cause is another important area of investigation.

Modification of the diet as a means of manipulating the gut microbiome has the advantage of being relatively easy and cheap to implement, and generally safe to do. However, it is uncertain whether diet modifications alone can result in a meaningful impact capable of inducing an anticancer effect. In addition, dietary changes are challenging to track and monitor given multiple confounders. Long term adherence is another limitation, although it is unclear whether prolonged dietary changes can induce permanent alterations in the diversity and composition of the gut microbiota [112]. There have been, however, recent efforts to address some of these limitations and more accurately predict the impact of dietary interventions. One of such examples, is the assessment of postprandial glycemic response (PPGR). Postprandial-associated hyperglycemia is associated with multiple medical conditions including cancer, and is highly variable amongst individuals. [113–115]. PPGR may be influenced by dietary habits, physical activity, and an individual's gut microbiome. Current methods to track PPGR (e.g. counting mealś carbohydrate content) have several limitations. Zeevi et al. recently devised a machine learning algorithm that integrates blood sampling, dietary habits, levels of physical activity, and gut microbiome analyses that can predict PPGR to food [113]. The authors reported significant associations between the standardized meal PPGRs of participants and both their clinical and gut microbiome data [116]. Next, the same authors conducted a personally tailored dietary intervention aimed to improve PPGRs, which resulted in significantly lower postprandial responses and consistent alterations to the composition of the gut microbiota. The incorporation of methods to assess patientś adherence to dietary interventions, such as PPGR analyses, should be encouraged in future studies.

Prebiotics

Prebiotics are substrates that serve as nutrients for beneficial microorganisms harbored by the host to promote health benefits. The majority of prebiotics are non-digestible carbohydrates such as fiber and resistant starch [110]. These are not degraded in the small intestine to further undergo fermentation by colonic resident microorganisms. Through this process, prebiotics are capable of modifying the gut microbiome favoring certain species [110]. Different prebiotic substances will favor the growth of specific species, inulin for example, a plant-based fructan, is shown to stimulate growth of *Faecalibacterium* and *Bifidobacterium* species. Both genera were associated with improved responses to ICI in melanoma patients [6]. Prebiotics are generally

regarded as safe and sold over the counter as dietary supplements. Akin to diet modifications, prebiotics are inexpensive and easy to implement. The same questions regarding diet modifications are also valid for prebiotics use. A potential limitation of their use is the fact that prebiotics, by merely stimulating the growth of select bacteria, assumes that the recipient already harbors those species colonized in their gut.

Probiotics

Probiotics are live bacteria and yeasts that, when administrated in a viable form and in adequate numbers, are putatively beneficial to health. Probiotics can be included in a variety of products, including foods, dietary supplements, or drugs. Typically, one to few bacteria strains are present in probiotic formulations. Probiotics have been extensively investigated in colorectal cancer patients and data show that the administration of probiotics containing strains of Lactobacillus acidophilus and B. lactis led to an increased abundance of butyrate-producing bacteria (particularly Faecalibacterium and other Clostridiales) within the tumor, and its associated non-tumor colonic mucosa and stool [117]. Another study assessed preoperative probiotic therapy on mucosal immunity in colorectal cancer patients, demonstrating altered cytokine profiles within the colonic mucosa assessed at the time of colonic resection, with lower IL-1β, IL-10, and IL-23A mRNA levels in the patients treated with probiotics compared to controls who received no probiotics [118].

Multiple studies are currently evaluating the role of probiotics as an adjuvant for ICI treated patients (NCT03829111, NCT04025307). On a cautionary note, Spencer et al. recently analyzed 113 patients with metastatic melanoma undergoing systemic treatment and reported that use of probiotics at baseline was associated with decreased microbiota diversity, which was associated with worse ICI responses [119]. This study also assessed baseline dietary habits, and found that patients with a high fiber diet were more likely to respond to ICI [119].

Fecal microbiota transplantation

FMT describes the process of transplanting (incompletely characterized) complex communities of microbes, metabolites, and other fecal materials from a healthy donor to a recipient. FMT has been successfully used to treat recurrent *Clostridioides difficile* infection, and ICI-induced steroid refractory colitis [85,120–122]. In preclinical models, tumor growth inhibition has been demonstrated in mice transplanted with stool from patients responding to ICI [1,6]. FMT is currently being investigated in several studies as an adjuvant of ICI treatments, across several malignancies (NCT03353402, NCT04264975, NCT03341143, NCT03772899, NCT04130763, NCT04116775, NCT04056026). FMT is also being explored to reduce and prevent treatment related toxicities (NCT04163289, NCT03772899, NCT03819296). Nonetheless, despite promising initial reports, the use of FMT has some caveats, including: 1) lack of process control, given bacteria strains composition is largely unknown and varies with stool donation; 2) lack of reproducible therapeutic stool at large scale; 3) safety concerns given the potential for transmission of known or unknown organisms as well as host-associated phenotypes. FMT has recently been linked to the death of two patients being treated for *Clostridioides difficile* colitis, due to the induction of antibiotics-resistant organisms, prompting the US Food and Drug Administration to issue a cautionary warning addressed to FMT researchers [123].

Microbial consortia and stool substitutes

Cultivated microbial consortia (groups of organisms grown together or separately) have been developed as an alternative to FMT and probiotics. Consortia are a defined mixture of pure live cultures of bacteria, often isolated from a stool sample of a healthy donor. They are designed to reproduce some of the complexities of more complete communities with fewer risks and greater reproducibility. Their design ranges from multi-species probiotics (essentially large numbers of individually selected species or strains co-administered, e.g. VE800, a designer probiotic assembled including 11 commensal species with ability to induce CD8+ responses), to cultivated "ecosystems" designed to enhance the engraftment of therapeutic species due to the inclusion of auxiliary taxa which satisfy taxonomic metabolic interdependencies [58]. The most complex of these consortia can be used as an alternative to FMT.

One such approach involves "microbial ecosystem therapeutics" which contains multiple individually characterized, human-derived bacterial strains purified and grown in conditions modeling that of the human distal gut, and has been successfully used to treat *Clostridioides difficile* infection [124]. A modified version of this ecosystem (MET4) is currently being tested in cancer patients receiving ICI both in the advanced and adjuvant setting (NCT03686202). METs have the advantage of combining the customizability, safety, reproducibility and scalable production of a probiotic, and the ecological and functional complexity of FMT. Our group recently presented preliminary data of 20 patients treated orally with MET4 while on ICI treatment, and demonstrated that MET4 was overall well tolerated, and that MET4 recipients were found to have an increased relative abundance of MET4-associated taxa, as well as a tendency in maintaining microbial diversity over time compared to controls in the advanced setting [125]. Further analyses are necessary to determine if such findings translate into clinical benefit for treated patients. Other stool substitutes such as SER-401 (NCT03817125) are also being investigated in combination with ICI.

Antibiotics

As discussed previously, there are mounting data associating the use of antibiotics prior to ICI treatments to decreased responses and survival. Nevertheless, to modulate the gut microbiome with antibiotics followed by the administration of FMT, probiotics or microbial consortia, which may facilitate the engraftment of desirable taxa, is an attractive strategy of modulating the microbiome to optimize clinical benefit. Preclinical studies suggest that antibiotic treated mice can have their microbiome restored post administration of FMT, or single bacteria strains. In humans, a recent trial conducted in patients undergoing stem cell transplantation, who are frequently treated with broad spectrum antibiotics and high dose chemotherapy agents, which significantly modify the composition of their microbiome, showed that autologous FMT can reconstitute their microbiota composition [73].

Currently, studies are ongoing utilizing antibiotics prior to introducing microbiome manipulations. For instance, the MCGRAW trial (NCT03817125) is an early phase study in patients with anti-PD1 therapy naïve, unresectable, or metastatic melanoma evaluating antibiotic pre-treatment with vancomycin to prime the gut microbiome for engraftment of SER-401. Optimal timing of antibiotic administration relative to immune-based therapy also needs to be delineated. Further carefully designed human studies are needed to clarify these questions.

As microbiome manipulations as adjuvants to ICI are a relatively novel field of investigation, the safety and tolerability of such interventions are under investigation. Fig. 3 speculates on safety and therapeutic effects of interventions. As a general rule, likely the more ecologically complex the intervention, the more safety concerns it poses, however, further studies are necessary to shed light on these questions.

Clinical trials investigating the microbiome

As the knowledge accumulates in the field of microbiome in oncology, opportunities emerge to interrogate this as a potential therapeutic strategy. Both proof-of-mechanism and proof-of-concept clinical trial design frameworks should be considered in ongoing efforts to incorporate microbiome research as an interventional strategy in human cancers. Proof-of-mechanism studies require a direct comparison of the host immune system and the tumor microenvironment before and after

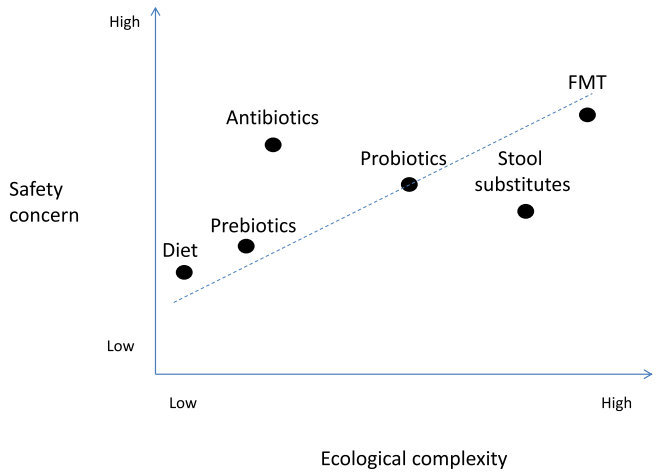

Fig. 3. Potential safety concerns and ecological complexity of microbiome modifying interventions. Higher complexity interventions, such as FMT, have the greatest potential safety issues.

any manipulation of the microbiome, while factoring in the impact of relevant variables such as the anticancer therapeutic, antibiotic usage and diet. The procurement of tumor tissues via needle biopsy or surgery is important in these studies in order to assess the immune contexture that may be altered by microbiome modulation. The preoperative window-of-opportunity setting is appealing for such proof-of-mechanism studies since the access to tumor tissues is straightforward, although the duration of microbial manipulation is limited and any delayed or long-term effects cannot be ascertained. The testing of microbiome modulation in the advanced disease settings may mitigate these pitfalls but the quantity of tumor tissues obtained by core needle biopsies maybe insufficient for extensive analyses. Regardless of the setting, a control arm without microbiome manipulation is informative to enable an objective assessment of clinical and molecular changes over time. It is critical that the clinicopathological and immunoprofiling examinations are performed in a blinded manner to avoid any bias. Importantly, all trials should incorporate longitudinal sampling of stool, blood, and tissue (whenever feasible). The longitudinal changes observed and their correlation with relevant clinical and molecular parameters will contribute to a better understanding of the complex interactions between the gut microbiome, host, tumor, treatment and toxicity.

Proof-of-concept studies focus on clinically meaningful outcomes such as objective tumor response, PFS or OS. These studies should only be conducted after the safety and tolerability of microbiome modulation have been confirmed. For patient populations receiving immuno-oncology treatments, randomized controlled studies of microbiome modulation in those who have primary or acquired resistance to immunotherapy would be of interest. It is likely impossible to control for confounders such as antibiotic needs and diet, but these data should be carefully collected to facilitate interpretation of results from clinical trials specifically designed to address the clinical utility of microbiome modulation.

In the near future, there will be an anticipated increase in the number of proof-of-mechanism and proof-of-concept clinical trials related to the microbiome in cancer. Standardizations related to sample collection and analysis, reporting of endpoints and confounders, and correlation of changes in the microbiome with clinical outcome must be urgently established to maximize knowledge gain in this emerging area.

Conclusions

Despite the burgeoning body of knowledge in the field of the microbiome as it relates to cancer pathogenesis and therapy, many unanswered questions remain that will require continued nonclinical and clinical investigations. The role of the microbiome in modulating response or resistance to local therapy such as radiotherapy, or systemic therapy including cytotoxic chemotherapy, targeted therapy and immunotherapy, and their related toxicity needs to be further elucidated given its therapeutic implications. Standardization of technical, methodological, analytical and reporting aspects is important to ensure validity and optimize comparability of research results. The differentiation of causality from association requires thoughtfully conceived evaluation in validated animal models as well as appropriately controlled clinical trials in patients. The joint efforts of the scientific community to collaborate in microbiome research and share data are critical to accelerate knowledge in this field.

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References

[1] Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillère R, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science 2018;359(6371):91–7.
[2] Killock D. Immunotherapy: gut bacteria modulate responses to PD-1 blockade. Nat Rev Clin Oncol 2018;15(1):6–7.
[3] Chaput N, Lepage P, Coutzac C, Soularue E, Le Roux K, Monot C, et al. Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. Ann Oncol 2017;28(6):1368–79.
[4] Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. Science 2015;350(6264):1084–9.
[5] Matson V, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre ML, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. Science 2018;359(6371):104–8.
[6] Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science 2018;359(6371):97–103.
[7] Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. Nature 2012;489(7415):231–41.
[8] Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. Science 2012;336(6086):1268.
[9] Zitvogel L, Galluzzi L, Viaud S, Vétizou M, Daillère R, Merad M, et al. Cancer and the gut microbiota: an unexpected link. Sci Transl Med 2015;7(271). 271ps1-ps1.
[10] Nogueira AR, Shoenfeld Y. Microbiome and autoimmune diseases: cause and effect relationship. Curr Opin Rheumatol 2019;31(5):471–4.
[11] Khosravi A, Mazmanian SK. Disruption of the gut microbiome as a risk factor for microbial infections. Curr Opin Microbiol 2013;16(2):221–7.
[12] Moore PS, Chang Y. Why do viruses cause cancer? Highlights of the first century of human tumour virology. Nat Rev Can 2010;10(12):878–89.
[13] Peek Jr RM, Blaser MJ. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Can 2002;2(1):28–37.
[14] Bonnet M, Buc E, Sauvanet P, Darcha C, Dubois D, Pereira B, et al. Colonization of the human gut by E. coli and colorectal cancer risk. Clin Can Res 2014;20(4):859–67.
[15] Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. Cell Host Microbe 2013;14(2):195–206.
[16] Zhang X, Liu Q, Liao Q, Zhao Y. Pancreatic cancer, gut microbiota, and therapeutic efficacy. J Can 2020;11(10):2749–58.
[17] Pushalkar S, Hundeyin M, Daley D, Zambirinis CP, Kurz E, Mishra A, et al. The pancreatic cancer microbiome promotes oncogenesis by induction of innate and adaptive immune suppression. Can Discov 2018;8(4):403–16.
[18] Hayes RB, Ahn J, Fan X, Peters BA, Ma Y, Yang L, et al. Association of oral microbiome with risk for incident head and neck squamous cell cancer. JAMA Oncol 2018;4(3):358–65.
[19] Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science (New York, NY). 2012;338(6103):120–3.
[20] Dzutsev A, Goldszmid RS, Viaud S, Zitvogel L, Trinchieri G. The role of the microbiota in inflammation, carcinogenesis, and cancer therapy. Eur J Immunol 2015;45(1):17–31.
[21] Song M, Chan AT, Sun J. Influence of the gut microbiome, diet, and environment on risk of colorectal cancer. Gastroenterology 2020;158(2):322–40.
[22] Wu J, Peters BA, Dominianni C, Zhang Y, Pei Z, Yang L, et al. Cigarette smoking and the oral microbiome in a large study of American adults. ISME J 2016;10(10):2435–46.

[23] Bullman S, Pedamallu CS, Sicinska E, Clancy TE, Zhang X, Cai D, et al. Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. Science 2017;358(6369):1443–8.
[24] Gomez A, Espinoza JL, Harkins DM, Leong P, Saffery R, Bockmann M, et al. Host genetic control of the oral microbiome in health and disease. Cell Host Microbe 2017;22(3). 269-78.e3.
[25] Goodman B, Gardner H. The microbiome and cancer. J Pathol 2018;244(5):667–76.
[26] Riquelme E, Zhang Y, Zhang L, Montiel M, Zoltan M, Dong W, et al. Tumor microbiome diversity and composition influence pancreatic cancer outcomes. Cell 2019;178(4):795–806.e12.
[27] Zhang L, Liu Y, Zheng HJ, Zhang CP. The oral microbiota may have influence on oral cancer. Front Cell Infect Microbiol 2019;9:476.
[28] Guerrero-Preston R, Godoy-Vitorino F, Jedlicka A, Rodríguez-Hilario A, González H, Bondy J, et al. 16S rRNA amplicon sequencing identifies microbiota associated with oral cancer, human papilloma virus infection and surgical treatment. Oncotarget 2016;7(32):51320–34.
[29] Guerrero-Preston R, White JR, Godoy-Vitorino F, Rodríguez-Hilario A, Navarro K, González H, et al. High-resolution microbiome profiling uncovers Fusobacterium nucleatum, Lactobacillus gasseri/johnsonii, and Lactobacillus vaginalis associated to oral and oropharyngeal cancer in saliva from HPV positive and HPV negative patients treated with surgery and chemo-radiation. Oncotarget 2017;8(67):110931–48.
[30] Curty G, de Carvalho PS, Soares MA. The role of the cervicovaginal microbiome on the genesis and as a biomarker of premalignant cervical intraepithelial neoplasia and invasive cervical cancer. Int J Mol Sci 2019;21(1).
[31] Cavarretta I, Ferrarese R, Cazzaniga W, Saita D, Lucianò R, Ceresola ER, et al. The microbiome of the prostate tumor microenvironment. Eur Urol 2017;72(4):625–31.
[32] Wu P, Zhang G, Zhao J, Chen J, Chen Y, Huang W, et al. Profiling the urinary microbiota in male patients with bladder cancer in China. Front Cell Infect Microbiol 2018;8:167.
[33] Yu T, Guo F, Yu Y, Sun T, Ma D, Han J, et al. Fusobacterium nucleatum promotes chemoresistance to colorectal cancer by modulating autophagy. Cell 2017;170(3). 548-63.e16.
[34] Yamamura K, Izumi D, Kandimalla R, Sonohara F, Baba Y, Yoshida N, et al. Intratumoral Fusobacterium nucleatum levels predict therapeutic response to neoadjuvant chemotherapy in esophageal squamous cell carcinoma. Clinical Cancer Research. 2019:clincanres.0318.2019.
[35] Mima K, Nishihara R, Qian ZR, Cao Y, Sukawa Y, Nowak JA, et al. Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. Gut 2016;65(12):1973–80.
[36] Gur C, Ibrahim Y, Isaacson B, Yamin R, Abed J, Gamliel M, et al. Binding of the Fap2 protein of Fusobacterium nucleatum to human inhibitory receptor TIGIT protects tumors from immune cell attack. Immunity 2015;42(2):344–55.
[37] Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe 2013;14(2):207–15.
[38] Geller LT, Barzily-Rokni M, Danino T, Jonas OH, Shental N, Nejman D, et al. Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug gemcitabine. Science 2017;357(6356):1156–60.
[39] Nejman D, Livyatan I, Fuks G, Gavert N, Zwang Y, Geller LT, et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. Science 2020;368(6494):973–80.
[40] Alexander JL, Wilson ID, Teare J, Marchesi JR, Nicholson JK, Kinross JM. Gut microbiota modulation of chemotherapy efficacy and toxicity. Nat Rev Gastroenterol Hepatol 2017;14(6):356–65.
[41] Reis Ferreira M, Andreyev HJN, Mohammed K, Truelove L, Gowan SM, Li J, et al. Microbiota- and radiotherapy-induced gastrointestinal side-effects (MARS) study: a large pilot study of the microbiome in acute and late-radiation enteropathy. Clin Cancer Res 2019.
[42] Gori S, Inno A, Belluomini L, Bocus P, Bisoffi Z, Russo A, et al. Gut microbiota and cancer: how gut microbiota modulates activity, efficacy and toxicity of antitumoral therapy. Crit Rev Oncol Hematol 2019;143:139–47.
[43] Iida N, Dzutsev A, Stewart CA, Smith L, Bouladoux N, Weingarten RA, et al. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science 2013;342(6161):967–70.
[44] Pflug N, Kluth S, Vehreschild JJ, Bahlo J, Tacke D, Biehl L, et al. Efficacy of antineoplastic treatment is associated with the use of antibiotics that modulate intestinal microbiota. Oncoimmunology 2016;5(6):e1150399.
[45] Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillère R, Hannani D, et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science 2013;342(6161):971–6.
[46] Fessler J, Matson V, Gajewski TF. Exploring the emerging role of the microbiome in cancer immunotherapy. J ImmunoTher Cancer 2019;7(1):108.
[47] Hou J, Zheng H, Li P, Liu H, Zhou H, Yang X. Distinct shifts in the oral microbiota are associated with the progression and aggravation of mucositis during radiotherapy. Radiother Oncol 2018;129(1):44–51.
[48] Frankel AE, Coughlin LA, Kim J, Froehlich TW, Xie Y, Frenkel EP, et al. Metagenomic shotgun sequencing and unbiased metabolomic profiling identify specific human gut microbiota and metabolites associated with immune checkpoint therapy efficacy in melanoma patients. Neoplasia 2017;19(10):848–55.
[49] Peters BA, Wilson M, Moran U, Pavlick A, Izsak A, Wechter T, et al. Relating the gut metagenome and metatranscriptome to immunotherapy responses in melanoma patients. Genome Med 2019;11(1):61.
[50] Wind TT, Gacesa R, Vich Vila A, de Haan JJ, Jalving M, Weersma RK, et al. Gut microbial species and metabolic pathways associated with response to treatment with immune checkpoint inhibitors in metastatic melanoma. Melanoma Res 2020;30(3):235–46.
[51] Vétizou M, Pitt JM, Daillère R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science (New York, NY) 2015;350(6264):1079–84.
[52] Scott AJ, Alexander JL, Merrifield CA, Cunningham D, Jobin C, Brown R, et al. International cancer microbiome consortium consensus statement on the role of the human microbiome in carcinogenesis. Gut 2019;68(9):1624–32.
[53] Haase S, Haghikia A, Wilck N, Müller DN, Linker RA. Impacts of microbiome metabolites on immune regulation and autoimmunity. Immunology 2018;154(2):230–8.
[54] Gurav A, Sivaprakasam S, Bhutia YD, Boettger T, Singh N, Ganapathy V. Slc5a8, a Na+-coupled high-affinity transporter for short-chain fatty acids, is a conditional tumour suppressor in colon that protects against colitis and colon cancer under low-fibre dietary conditions. Biochem J 2015;469(2):267–78.
[55] Uribe-Herranz M, Bittinger K, Rafail S, Guedan S, Pierini S, Tanes C, et al. Gut microbiota modulates adoptive cell therapy via CD8α dendritic cells and IL-12. JCI. Insight 2018;3(4).
[56] Morton AM, Sefik E, Upadhyay R, Weissleder R, Benoist C, Mathis D. Endoscopic photoconversion reveals unexpectedly broad leukocyte trafficking to and from the gut. Proc Natl Acad Sci USA 2014;111(18):6696–701.
[57] Wang F, Yin Q, Chen L, Davis MM. Bifidobacterium can mitigate intestinal immunopathology in the context of CTLA-4 blockade. Proc Natl Acad Sci USA 2018;115(1):157–61.
[58] Tanoue T, Morita S, Plichta DR, Skelly AN, Suda W, Sugiura Y, et al. A defined commensal consortium elicits CD8 T cells and anti-cancer immunity. Nature 2019;565(7741):600–5.
[59] Mager LF, Burkhard R, Pett N, Cooke NCA, Brown K, Ramay H, et al. Microbiome-derived inosine modulates response to checkpoint inhibitor immunotherapy. Science 2020;369(6510):1481–9.
[60] Soldati L, Di Renzo L, Jirillo E, Ascierto PA, Marincola FM, De Lorenzo A. The influence of diet on anti-cancer immune responsiveness. J Transl Med 2018;16(1):75.
[61] Derosa L, Hellmann MD, Spaziano M, Halpenny D, Fidelle M, Rizvi H, et al. Negative association of antibiotics on clinical activity of immune checkpoint inhibitors in patients with advanced renal cell and non-small-cell lung cancer. Ann Oncol 2018;29(6):1437–44.
[62] Peng W, Chen JQ, Liu C, Malu S, Creasy C, Tetzlaff MT, et al. Loss of PTEN promotes resistance to T cell-mediated immunotherapy. Can Discov 2016;6(2):202–16.
[63] Mielgo-Rubio X, Chara L, Sotelo-Lezama M, Lopez Castro R, Rubio-Martínez J, Velastegui A, et al. MA10.01 Antibiotic Use and PD-1 Inhibitors: Shorter Survival in Lung Cancer, Especially When Given Intravenously. Type of Infection Also Matters. Journal of Thoracic Oncology. 2018;13(10, Supplement):S389.
[64] Lalani AA, Xie W, Braun DA, Kaymakcalan M, Bossé D, Steinharter JA, et al. Effect of antibiotic use on outcomes with systemic therapies in metastatic renal cell carcinoma. Eur Urol Oncol 2020;3(3):372–81.
[65] Abu-Sbeih H, Herrera LN, Tang T, Altan M, Chaftari A-MP, Okhuysen PC, et al. Impact of antibiotic therapy on the development and response to treatment of immune checkpoint inhibitor-mediated diarrhea and colitis. Journal for ImmunoTherapy of Cancer. 2019;7(1) :242.
[66] Wilson BE, Routy B, Nagrial A, Chin VT. The effect of antibiotics on clinical outcomes in immune-checkpoint blockade: a systematic review and meta-analysis of observational studies. Can Immunol Immunother 2020;69(3):343–54.
[67] Palleja A, Mikkelsen KH, Forslund SK, Kashani A, Allin KH, Nielsen T, et al. Recovery of gut microbiota of healthy adults following antibiotic exposure. Nat Microbiol 2018;3(11):1255–65.
[68] Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 2008;6(11):e280.
[69] Ahmed J, Kumar A, Parikh K, Anwar A, Knoll BM, Puccio C, et al. Use of broad-spectrum antibiotics impacts outcome in patients treated with immune checkpoint inhibitors. Oncoimmunology. 2018;7(11) :e1507670-e.
[70] Tinsley N, Zhou C, Tan G, Rack S, Lorigan P, Blackhall F, et al. Cumulative antibiotic use significantly decreases efficacy of checkpoint inhibitors in patients with advanced cancer. Oncologist 2020;25(1):55–63.
[71] Jenq RR, Taur Y, Devlin SM, Ponce DM, Goldberg JD, Ahr KF, et al. Intestinal blautia is associated with reduced death from graft-versus-host disease. Biol Blood Marrow Transplant 2015;21(8):1373–83.
[72] Weber D, Oefner PJ, Hiergeist A, Koestler J, Gessner A, Weber M, et al. Low urinary indoxyl sulfate levels early after transplantation reflect a disrupted microbiome and are associated with poor outcome. Blood 2015;126(14):1723–8.
[73] Taur Y, Jenq RR, Perales MA, Littmann ER, Morjaria S, Ling L, et al. The effects of intestinal tract bacterial diversity on mortality following allogeneic hematopoietic stem cell transplantation. Blood 2014;124(7):1174–82.
[74] Dubin K, Callahan MK, Ren B, Khanin R, Viale A, Ling L, et al. Intestinal microbiome analyses identify melanoma patients at risk for checkpoint-blockade-induced colitis. Nat Commun 2016;7:10391.
[75] Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell 2014;157(1):121–41.
[76] Belkaid Y, Harrison OJ. Homeostatic immunity and the microbiota. Immunity 2017;46(4):562–76.
[77] Chu H, Mazmanian SK. Innate immune recognition of the microbiota promotes host-microbial symbiosis. Nat Immunol 2013;14(7):668–75.

[78] Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. Nat Rev Immunol 2016;16(6):341–52.
[79] Fluckiger A, Daillère R, Sassi M, Sixt BS, Liu P, Loos F, et al. Cross-reactivity between tumor MHC class I-restricted antigens and an enterococcal bacteriophage. Science 2020;369(6506):936–42.
[80] Gil-Cruz C, Perez-Shibayama C, De Martin A, Ronchi F, van der Borght K, Niederer R, et al. Microbiota-derived peptide mimics drive lethal inflammatory cardiomyopathy. Science 2019;366(6467):881–6.
[81] Blank CU, Haanen JB, Ribas A, Schumacher TN. CANCER IMMUNOLOGY. The "cancer immunogram". Science 2016;352(6286):658–60.
[85] Wang Y, Wiesnoski DH, Helmink BA, Gopalakrishnan V, Choi K, DuPont HL, et al. Fecal microbiota transplantation for refractory immune checkpoint inhibitor-associated colitis. Nat Med 2018;24(12):1804–8.
[86] Bartram AK, Lynch MD, Stearns JC, Moreno-Hagelsieb G, Neufeld JD. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. Appl Environ Microbiol 2011;77(11):3846–52.
[87] Banos S, Lentendu G, Kopf A, Wubet T, Glöckner FO, Reich M. A comprehensive fungi-specific 18S rRNA gene sequence primer toolkit suited for diverse research issues and sequencing platforms. BMC Microbiol 2018;18(1):190.
[88] Hillmann B, Al-Ghalith GA, Shields-Cutler RR, Zhu Q, Gohl DM, Beckman KB, et al. Evaluating the Information Content of Shallow Shotgun Metagenomics. mSystems. 2018;3(6) :e00069-18.
[89] Aguiar-Pulido V, Huang W, Suarez-Ulloa V, Cickovski T, Mathee K, Narasimhan G. Metagenomics, metatranscriptomics, and metabolomics approaches for microbiome analysis. Evol Bioinform Online 2016;12(Suppl 1):5–16.
[90] Lau JT, Whelan FJ, Herath I, Lee CH, Collins SM, Bercik P, et al. Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. Genome Med 2016;8(1):72.
[91] Lagier JC, Armougom F, Million M, Hugon P, Pagnier I, Robert C, et al. Microbial culturomics: paradigm shift in the human gut microbiome study. Clin Microbiol Infect 2012;18(12):1185–93.
[92] Jian C, Luukkonen P, Yki-Järvinen H, Salonen A, Korpela K. Quantitative PCR provides a simple and accessible method for quantitative microbiota profiling. PLoS ONE 2020;15(1):e0227285.
[93] Allaband C, McDonald D, Vázquez-Baeza Y, Minich JJ, Tripathi A, Brenner DA, et al. Microbiome 101: studying, analyzing, and interpreting gut microbiome data for clinicians. Clin Gastroenterol Hepatol 2019;17(2):218–30.
[94] Peled JU, Devlin SM, Staffas A, Lumish M, Khanin R, Littmann ER, et al. Intestinal microbiota and relapse after hematopoietic-cell transplantation. J Clin Oncol 2017;35(15):1650–9.
[95] Ji BW, Sheth RU, Dixit PD, Tchourine K, Vitkup D. Macroecological dynamics of gut microbiota. Nat Microbiol 2020;5(5):768–75.
[96] Hornung B, Martins Dos Santos VAP, Smidt H, Schaap PJ. Studying microbial functionality within the gut ecosystem by systems biology. Genes Nutr 2018;13(5).
[97] Lee CH, Belanger JE, Kassam Z, Smieja M, Higgins D, Broukhanski G, et al. The outcome and long-term follow-up of 94 patients with recurrent and refractory Clostridium difficile infection using single to multiple fecal microbiota transplantation via retention enema. Eur J Clin Microbiol Infect Dis 2014;33(8):1425–8.
[98] Gopalakrishnan V, Helmink BA, Spencer CN, Reuben A, Wargo JA. The influence of the gut microbiome on cancer, immunity, and cancer immunotherapy. Can Cell 2018;33(4):570–80.
[99] Sinha R, Abnet CC, White O, Knight R, Huttenhower C. The microbiome quality control project: baseline study design and future directions. Genome Biol 2015;16:276.
[100] Kim D, Hofstaedter CE, Zhao C, Mattei L, Tanes C, Clarke E, et al. Optimizing methods and dodging pitfalls in microbiome research. Microbiome 2017;5(1):52.
[101] Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol 2014;12:87.
[102] Suez J, Zmora N, Zilberman-Schapira G, Mor U, Dori-Bachash M, Bashiardes S, et al. Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous FMT. Cell 2018;174(6). 1406-23.e16.
[103] Smillie CS, Sauk J, Gevers D, Friedman J, Sung J, Youngster I, et al. Strain tracking reveals the determinants of bacterial engraftment in the human gut following fecal microbiota transplantation. Cell Host Microbe 2018;23(2). 229-40.e5.
[104] Walter J, Maldonado-Gómez MX, Martínez I. To engraft or not to engraft: an ecological framework for gut microbiome modulation with live microbes. Curr Opin Biotechnol 2018;49:129–39.
[105] Nguyen TL, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? Dis Model Mech 2015;8(1):1–16.
[106] Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature 2013;500(7464):541–6.
[107] Khan MAW, Ologun G, Arora R, McQuade JL, Wargo JA. Gut microbiome modulates response to cancer immunotherapy. Dig Dis Sci 2020;65(3):885–96.
[108] De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, et al. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut 2016;65(11):1812–21.
[109] Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science (New York, NY). 2011;334(6052):105–8.
[110] Singh RK, Chang H-W, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. J Translat Med 2017;15(1):73.
[111] Trompette A, Gollwitzer ES, Pattaroni C, Lopez-Mejia IC, Riva E, Pernot J, et al. Dietary fiber confers protection against flu by shaping Ly6c(-) patrolling monocyte hematopoiesis and CD8(+) T cell metabolism. Immunity 2018;48(5):992–1005 e8.
[112] Leeming ER, Johnson AJ, Spector TD, Le Roy CI. Effect of diet on the gut microbiota: rethinking intervention duration. Nutrients 2019;11(12):2862.
[113] Zeevi D, Korem T, Zmora N, Israeli D, Rothschild D, Weinberger A, et al. Personalized nutrition by prediction of glycemic responses. Cell 2015;163(5):1079–94.
[114] Lamkin DM, Spitz DR, Shahzad MMK, Zimmerman B, Lenihan DJ, Degeest K, et al. Glucose as a prognostic factor in ovarian carcinoma. Cancer 2009;115(5):1021–7.
[115] Blaak EE, Antoine JM, Benton D, Björck I, Bozzetto L, Brouns F, et al. Impact of postprandial glycaemia on health and prevention of disease. Obes Rev 2012;13(10):923–84.
[116] Xiao S, Fei N, Pang X, Shen J, Wang L, Zhang B, et al. A gut microbiota-targeted dietary intervention for amelioration of chronic inflammation underlying metabolic syndrome. FEMS Microbiol Ecol 2014;87(2):357–67.
[117] Hibberd AA, Lyra A, Ouwehand AC, Rolny P, Lindegren H, Cedgård L, et al. Intestinal microbiota is altered in patients with colon cancer and modified by probiotic intervention. BMJ Open Gastroenterol 2017;4(1):e000145.
[118] Consoli MLD, da Silva RS, Nicoli JR, Bruña-Romero O, da Silva RG, de Vasconcelos Generoso S, et al. Randomized clinical trial. J Parent Enteral Nutrit 2016;40(8):1114–21.
[119] Spencer CN, Gopalakrishnan V, McQuade J, Andrews MC, Helmink B, Khan MAW, et al. Abstract 2838: The gut microbiome (GM) and immunotherapy response are influenced by host lifestyle factors. Cancer Research. 2019;79(13 Supplement):2838.
[120] Smits LP, Bouter KE, de Vos WM, Borody TJ, Nieuwdorp M. Therapeutic potential of fecal microbiota transplantation. Gastroenterology 2013;145(5):946–53.
[121] Kelly CR, Khoruts A, Staley C, Sadowsky MJ, Abd M, Alani M, et al. Effect of fecal microbiota transplantation on recurrence in multiply recurrent clostridium difficile infection: a randomized trial. Ann Intern Med 2016;165(9):609–16.
[122] Juul FE, Garborg K, Bretthauer M, Skudal H, Øines MN, Wiig H, et al. Fecal microbiota transplantation for primary clostridium difficile infection. N Engl J Med 2018;378(26):2535–6.
[123] McSeveney. M. FDA In Brief: FDA warns about potential risk of serious infections caused by multi-drug resistant organisms related to the investigational use of Fecal Microbiota for Transplantation. 2019(FDA In Brief).
[124] Petrof EO, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, et al. Stool substitute transplant therapy for the eradication of Clostridium difficile infection: 'RePOOPulating' the gut. Microbiome 2013;1(1):3.
[125] Araujo DV, Bernal MO, Tan TJY, Heirali AA, Schneeberger PHH, Muniz TP, et al. First-in-class microbial ecosystem therapeutics 4 (MET4) in metastatic solid cancer patients treated with immunotherapy: MET4-IO. Journal of Clinical Oncology. 2020;38(15_suppl):3098.