# Facts and Hopes for Gut Microbiota Interventions in Cancer Immunotherapy

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## ABSTRACT

Immune checkpoint inhibitors (ICI) targeting cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) proteins transformed the management of advanced cancers. Many tumor-intrinsic factors modulate immunological and clinical responses to such therapies, but ample evidence also implicates the gut microbiome in responses. The gut microbiome, comprising the bacteria, archaea, fungi, and viruses that live in the human digestive tract, is an established determinant of host immunity, but its impact on response to ICI therapy in mice and humans with cancer has only recently been appreciated. Therapeutic interventions to optimize microbiota composition to improve immunotherapy outcomes show promise in mice and humans with cancer. In this review, we discuss the rationale for gut microbiome–based cancer therapies, the results from early-phase clinical trials, and possible future developments.

## Introduction

Tumor antigen-specific cytotoxic T cells are present in advanced cancers but often fail to induce tumor rejection (1–3) because cancer cells utilize many tumor-intrinsic mechanisms to escape immune-mediated destruction. Among these, the programmed death 1/programmed death ligand 1 (PD-1/PD-L1) axis is a key player in the inhibition of cytotoxic $CD8^+$ T cells, and immune checkpoint inhibitors (ICI) targeting PD-L1 promote effective and often durable responses in patients with a variety of cancer types, including melanoma, non–small cell lung cancer (NSCLC), and renal cell carcinoma (RCC; refs. 4–6). Biomarkers of response to PD-L1 blockade include $CD8^+$ tumor-infiltrating lymphocytes (TIL; refs. 7, 8), IFNγ gene expression (9, 10), high tumor mutation burden (TMB; refs. 10–12), or PD-L1 expression on tumor or T cells (5, 13). Strikingly, multiple studies support the role of the gut microbiome in regulating clinical responses to ICI in various preclinical models and in patients with cancer. In this review, we (i) summarize preclinical and clinical data supporting the role of the gut microbiome in determining response to cancer immunotherapy; (ii) describe the spectrum of therapeutic strategies to target the microbiome in cancer and their present status; and (iii) present key unanswered questions to address in ongoing and future research.

## The Gut Microbiome in Cancer Immunotherapy

### Preclinical data implicating gut microbiome composition in cancer immunotherapy

Evidence supports the role of gut commensals in mediating the efficacy of anticancer therapies, including chemotherapy, radiotherapy, and immunotherapies, in mouse tumor models. For example, cyclophosphamide induces gut transmucosal translocation of *Enterococcus hirae* to secondary lymphoid organs with consequent stimulation of Th17 responses and IFN-producing $CD8^+$ T-effector cells in sarcoma models and lung adenocarcinoma models (14, 15). In addition, commensal bacteria mediate oxaliplatin-induced immunogenic cell death by modulating reactive oxygen species (ROS; ref. 16) and the adjuvanticity of ileal bacteria (*Erysipelotrichaceae* spp., *Bacteroides fragilis*) that dictate the balance between antitumor T-follicular helper (Tfh) cells and deleterious Th17 responses in colon cancer (17). The gut microbiome also mediates the effects of hypofractionated radiotherapy on irradiated and nonradiated tumors in an IFNγ and cytotoxic T-cell–dependent manner in melanoma and human papillomavirus (HPV)-driven cancer models (18). Notably, the efficacy of adoptive cellular therapy (ACT) following total body irradiation (TBI) depends on lipopolysaccharide (LPS) and host Toll-like receptor 4 (TLR4) signaling mediated by bacterial translocation (19, 20). Systemically administered *Bifidobacterium* spp. can accumulate in tumors and improves anti-CD47 immunotherapy in a stimulator of IFN genes (STING) and IFN-dependent fashion (21). In addition, anticytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) efficacy depends on distinct *Bacteroides* spp., and broad-spectrum antibiotic treatment or housing in germ-free facilities attentuates the efficacy of CTLA-4 blockade in mice bearing MCA205 fibrosarcoma. In such mice, anti-CTL4 efficacy is partially restored after treatment with oral vancomycin, oral gavage with *B. fragilis*, immunization with *B. fragilis* polysaccharides, or adoptive transfer of *B. fragilis*–specific Th1 cells (22). Further, anti–CTLA-4 therapy appears to directly perturb the gut microbiome, specifically causing reductions in *Bacteroidales* and *Burkholderiales* and increases in *Clostridiales* spp. (22). Similarly, the efficacy of PD-1 blockade depends on *Bifidobacterium* spp. that act upon host dendritic cells (DC) to augment T-cell function in preclinical models of melanoma (23). Interestingly, *E. faecium*, but not *E. faecalis*, contains peptidoglycan hydrolase secreted antigen A (SagA) that generates nucleotide-binding oligomerization domain 2 (NOD2)-active muropeptides able to modulate anti–PD-1 treatment efficacy *in vivo* (24).

Finally, emerging evidence indicates that carcinogenesis induces regenerating islet-derived protein 3 γ (Reg3-γ)-mediated β-adrenergic receptor-dependent epithelial permeability and, consequently, *Clostridium* spp.-dominant dysbiosis. This finding implicates the gut microbiota in carcinogenesis and cancer progression (25). Collectively, these data provide compelling preclinical evidence that gut microbiota

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mediate the efficacy of a range of anticancer therapies, including chemotherapy, radiotherapy, and immunotherapy.

## Human data implicating gut microbiome composition in cancer immunotherapy

Multiple observations underscore the role of gut microbiota in determining response in ICI-treated patients. Firstly, in line with preclinical studies that showed that broad-spectrum antibiotics promoted unfavorable clinical outcomes to treatment with ICI targeting CTLA-4 (22) or PD-1/PD-L1 (26–28), patients with cancer who received broad-spectrum antibiotics shortly before or during ICI therapy had poorer progression-free survival (PFS) and overall survival compared with those who did not receive antibiotics (29, 30). These observations suggest that a diverse gut microbiota plays a critical role in promoting antitumor immunity (29, 31–34). Second, studies of the gut microbiota composition of ICI-treated patients with melanoma (26, 27, 35–37), NSCLC (28, 38–40), RCC (28, 41), and gastrointestinal (GI) cancers (42) reveal higher levels of distinct bacterial taxa in ICI-responders (R) compared with non-responders (NR; **Table 1**). These studies identified specific members of each of the major bacterial phyla colonizing humans—Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, and Verrucomicrobia—in patients who derived durable benefit from PD-1, CTLA-4, or dual PD-1/CTLA-4 blockade (**Table 1**). Conversely, the gut microbiota of NRs exhibited increased abundance of genera *Bacteroides* (phylum Bacteroidetes) and *Ruminococcus* and *Roseburia* (both Firmicutes; **Table 1**). While individually significant, these studies had a striking feature; each largely identified different sets of microbes. Notably, while increased alpha diversity was correlated with improved outcome in early studies (26), evidence for an association with ICI benefit is lacking.

The startling lack of consensus regarding the microbial signals associated with clinical response raises many questions about the significance of these findings. Multiple factors may impede the interpretation of the data linking ICI and gut microbiome composition, including the small sizes of individual cohorts, variable definitions of clinical response, distinct bioinformatic analytic pipelines, and the many confounding factors influencing gut microbiome composition (diet, treatment, geography, ethnicity, etc.) (highlighted in **Table 1**). To determine the effect of analytic pipelines upon observed taxa disparity, two studies utilized a consistent bioinformatic approach for taxonomic assignment with appropriate statistical corrections and analyzed raw sequencing data from multiple published datasets (43, 44). Gharaibeh and colleagues determined that the Gopalakrishnan and colleagues, Matson and colleagues, and Routy and colleagues datasets each had a unique microbial 16S rDNA signal and that there were shared orthologues in pairwise comparisons. However, there was a lack of common orthologues in all three datasets, suggesting that differences in analytic pipelines could not explain the signal differences across studies and that models based on microbial gene composition (function) rather than community composition (form) may have better predictive power (43). Separately, Shaikh and colleagues developed biomarkers of clinical outcome to ICIs (R index and NR index, respectively) based on gut microbial signatures in Rs and NRs, and concluded that the NR-index outperformed the R-index and literature-based indices in predicting outcome to ICI therapy in both a random effects model and a sensitivity/specificity analysis (44). Population-specific differences exert important effects in microbiome studies (45, 46) that may be overcome by using large datasets comprising multiple cohorts and performing intra-cohort and cross-cohort comparisons to yield better combined-cohort prediction. Also, ICI therapy, in particular, can yield novel patterns of response, meaning that time-to-event analyses evaluating a landmark timepoint [1 year PFS rather than objective response rate (ORR)] may better define the magnitude of benefit and the impact of a predictive biomarker (47, 48). To test whether a large dataset could overcome population-specific differences, Lee and colleagues used source-level metadata and a machine learning framework to evaluate normalized baseline metagenomic data from five new observational cohorts ($n = 165$) and four previously described cohorts ($n = 147$) of patients with ICI-naïve melanoma, and determined how combined taxonomic and functional microbiome signatures performed against radiographic assessment of response (49). The authors found cohort-specific microbial signatures. However, neither taxonomic nor functional microbiome biomarkers identified consistent signals across cohorts, suggesting that even unbiased machine learning methods are not sufficient to overcome between-cohort and microbiota differences. This fact is particularly apt when cohorts are small and/or between-cohort differences are significant (49). To determine the role of time-to-event analyses in predicting how microbiome-based signatures affect ICI outcome, McCulloch and colleagues evaluated a melanoma cohort treated with PD-1 monotherapy, along with four published datasets, and utilized PFS—rather than radiographic response—to infer PD-1 benefit (50). They found that baseline microbiota composition was optimally associated with clinical outcome at approximately 1 year after initiating treatment. Meta-analysis and other bioinformatic analyses of the combined data across cohorts revealed that bacteria associated with specific microbiota signatures correlated with favorable (Lachnospiraceae and Ruminococcaceae families of Firmicutes phylum and Actinobacteria phylum) or unfavorable (members of Bacteroidetes and Proteobacteria phyla) outcomes to PD-1 ICI therapy (50). These findings provide a scientific rationale for selecting the optimal endpoint (time-to-event rather than ORR) for inferring how baseline gut microbiome composition affects ICI response. Together with the ability of fecal microbiome transplantation (FMT) to overcome primary PD-1 resistance (51), they suggest that the early response is dominated by host-intrinsic and tumor-intrinsic factors but early nonresponse may be determined by the gut microbiota composition. This effect wanes in significance late in treatment when adaptive resistance mechanisms dominate.

Evidence suggests that human gut microbiota comprise many discrete ecologically balanced communities—"enterotypes" or "enteric microbiotypes"—that tend to be temporally resilient but still modifiable by diet, drugs, and lifestyle (52–57). Despite considerable effort to establish the link between the composition of individual taxa to PD-1 ICI response, the relationship between distinct enteric microbiotypes and this response remains poorly understood. McCulloch and colleagues mapped patient-level metagenomic data onto a microbiome map derived from an American Gut Project database of more than 10,000 fecal samples from across the United States and found that the distinct enteric microbiotypes of different geographical areas accounted for most of the between-cohort differences. This finding suggests that enteric microbiotypes could be accurately identified in large datasets despite being not entirely discrete and varying tremendously by taxonomic, functional, and ecologic properties (58). These data remain to be validated in larger series but still underscore the utility of such classifiers in developing microbiota-based diagnostics and/or therapeutic interventions. This work further helps to illuminate how strain-level population structure and genetic diversity contribute to immunotherapy outcome (58–60).

Collectively, these observations underscore the need to perform studies with large and deeply annotated datasets, clinically meaningful endpoints, geographically distinct populations, and stool samples collected and processed in a uniform fashion. Whether the findings

**Table 1.** Published studies evaluating gut microbiome composition in patients with advanced cancer treated with ICI.

| Study reference | Histology | ICI | Geographic location of samples | Sample size | Collection timepoint | Responder definition and timepoint | Sequencing method; taxonomic assignment | Bacterial taxa associated with improved benefit (study-specific; top 10) | Association between alpha diversity metrics and outcome |
|---|---|---|---|---|---|---|---|---|---|
| Chaput and colleagues (35) | Melanoma | Anti–CTLA-4 (ipilimumab) | Villejuif, France | 26 (9 R, 17 NR) | Pre-, and on-treatment (all paired) | Long-term clinical benefit (tumor shrinkage ≥50% relative to baseline; or tumor <50% shrinkage relative to baseline but <25% increase relative to nadir); 6 months | 16S; QIIME | *Faecalibacterium* spp.; *Gemmiger* spp. | Diversity metrics were not associated with outcome. Decreased diversity (lower Shannon index) was associated with colitis. |
| Frankel and colleagues (36) | Melanoma | Anti–PD-1 (nivolumab or pembrolizumab), anti–CTLA-4 (ipilimumab), and ipilimumab/ nivolumab | Dallas, TX | 39 (24 R, 15 NR) | Pre- | Radiographic response per RECIST v1.1; 2–3 months | MSS; MetaPhlAn | *Bacteroides caccae*; *Streptococcus parasanguinis*; PD-1 patients: *Dorea formicigenerans*, PD-1/CTLA-4 patients: *Faecalibacterium prausnitzii, Holdemania filiformis* | Diversity metrics were not associated with outcome. |
| Gopalakrishnan and colleagues (26) | Melanoma | Anti–PD-1 (nivolumab or pembrolizumab) | Houston, TX | 43 (30 R, 13 NR) | Pre- (41), and on- (2) treatment | Radiographic response per RECIST v1.1; 6 months | 16S; BLAST and mothur | *Firmicutes* spp., *Clostridia* spp., *Ruminococcaceae* spp., *F. prausnitzii*, *R. bromii*, *Porphyromonas pasteri*, *Veilonellaceae* spp., C. *hungatei*, *Phascolarctobacterium* spp., *P. faecium* | Increased diversity (higher inverse Simpson) was associated with improved outcome. |
| Matson and colleagues (27) | Melanoma | Anti–PD-1 (nivolumab or pembrolizumab), anti–CTLA-4 (ipilimumab) | Chicago, IL | 42 (16R, 26 NR) | Pre- | Radiographic response per RECIST v1.1; not stated | 16S; QIIME MSS; MetaPhlAn2 | *Bifidobacteriaceae* spp., *Coriobacteriaceae* spp., *Lachospiraceae* spp., *Lactobacillaceae* spp., *Enterococcaceae* spp., *Enterobacteriaceae* spp., *Porphyromonadaceae* spp. | Diversity metrics were not associated with outcome. |
| Peters and colleagues (37) | Melanoma | Anti–PD-1 (nivolumab or pembrolizumab), anti–CTLA-4 (ipilimumab), and ipilimumab/ nivolumab | New York, NY | 27 | Pre- | PFS | 16S; QIIME MSS; MetaPhlAn2 | *Faecalibacterium prausnitzii, Coprococcus eutactus, Prevotella stercorea, Streptococcus sanguinis, Streptococcus anginosus, and Lachnospiraceae bacterium* 3 1 46FAA | Increased diversity (higher Shannon) was associated with improved outcome in 16S data but not in MSS data. |

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**Table 1.** Published studies evaluating gut microbiome composition in patients with advanced cancer treated with ICI. (Cont'd)

| Study reference | Histology | ICI | Geographic location of samples | Sample size | Collection timepoint | Responder definition and timepoint | Sequencing method; taxonomic assignment | Bacterial taxa associated with improved benefit (study-specific; top 10) | Association between alpha diversity metrics and outcome |
|---|---|---|---|---|---|---|---|---|---|
| Routy and colleagues (28) | RCC, NSCLC | Anti–PD-1 (nivolumab or pembrolizumab) | Villejuif, France | 153 (86 R, 67 NR) Discovery: NSCLC 60 (31 R, 29 NR); RCC (25R, 15NR) Validation: NSCLC 27 (10 R, 17 NR); RCC 26 (20 R, 6 NR) | Pre-, and on-treatment (all paired) | Best radiographic response per RECIST v1.1 (including SD); 3-month PFS | MSS; METEOR and MetaOMineR | Radiographic response: *A. muciniphila*, *Firmicutes* spp., *Eubacterium* spp., *Lachnospiraceae* spp., *Erysipelotrichaceae* spp., *Cloacibacillus porcorum*, *E. faecium*, *Intestinimonas* spp., *Clostridiales* spp., *Alistipes* spp. Improved PFS: *Firmicutes* spp., *Eubacterium* spp., *Alistipes* spp., *A. muciniphila*, *Intestinimonas* spp., *B. nordii*, *Bacteroides xylanisolvens*, *Blautia* spp., *Lachnospiraceae* spp., *Clostridiales* spp. | Diversity metrics were not associated with outcome. |
| Jin and colleagues (38) | NSCLC | Anti–PD-1 nivolumab | Shanghai, China | 37 (23 R, 14 NR) | Pre- | Best radiographic response per RECIST v1.1 (including SD ≥6 months) | 16S; QIIME | *Ruminococcus* spp., *A. putredinis, P. copri, B. longum, Clostridiales* spp., *Parabacteroides* spp., *Shigella* spp. | Increased diversity (higher Shannon and inverse Simpson) was associated with improved outcome. |
| Hakozaki and colleagues (39) | NSCLC | Anti–PD-(L)1 (nivolumab, pembrolizumab, or atezolizumab) | Tokyo, Japan | 70 (24 R[a], 27 NR) | Pre- | Best radiographic response per RECIST v1.1 (including SD ≥6 months); PFS | 16S; SILVA | *Ruminococcaceae* UCG 13 and *Agathobacter* spp. | Diversity metrics were not associated with outcome. Antibiotic use was associated with reduced diversity metrics (Shannon and inverse Simpson). |
| Derosa and colleagues (40) | NSCLC | Anti–PD-(L)1 (nivolumab, pembrolizumab or atezolizumab) singly or in combination with chemotherapy in either first- or second-line NSCLC | Villejuif, France | 338 patients (R - 28% $Akk^+$ and 18% $Akk^-$; NR - 44% $Akk^+$ and 50% $Akk^-$) | Pre- (V1), and early on-treatment (V2) | Best radiographic response per RECIST v1.1 | MSS; MetaOMineR and MetaPhlAn3 | *Akkermansia muciniphila* | Increased diversity (higher Shannon) was associated with improved outcome. |
| Derosa and colleagues (41) | RCC | PD-1 (nivolumab) treated in NIVOREN GETUG-AFU 26 phase II study | Villejuif, France | 58 (30 R, 28 NR) after exclusion of 11 patients who received antibiotics | Pre- | Best radiographic response per RECIST v1.1 (including SD ≥12 months); PFS | MSS; MetaOMineR and MetaPhlAn2 | *A. muciniphila*, *Eubacterium* spp., *Firmicutes bacterium* CAG_124, *Methanobrevibacter smithii*, *Firmicutes bacterium* CAG_103, *Dialister succinatiphilus YIT_11850*, *Eubacterium siraeum CAG_80*, *Bacteroides cellulosilyticus*, *Acinetobacter* spp. CAG_196, *Faecalibacterium* spp. CAG_74 | Diversity metrics were not associated with outcome. Antibiotic use was associated with reduced beta diversity (ANOSIM). |

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**Table 1.** Published studies evaluating gut microbiome composition in patients with advanced cancer treated with ICI. (Cont'd)

| Study reference | Histology | ICI | Geographic location of samples | Sample size | Collection timepoint | Responder definition and timepoint | Sequencing method; taxonomic assignment | Bacterial taxa associated with improved benefit (study-specific; top 10) | Association between alpha diversity metrics and outcome |
|---|---|---|---|---|---|---|---|---|---|
| Peng and colleagues (42) | GI (including esophageal, gastric, colorectal, and others) | Anti–PD-(L)1 (nivolumab, pembrolizumab, or atezolizumab) or anti–PD-1/anti–CTLA-4 combination (ipilimumab with nivolumab) | Beijing, China | 74 (45 R, 29 NR) | Pre-, and on-treatment | Best radiographic response per RECIST v1.1 (including SD ≥3 months); PFS | 16S; SILVA and HUMAnN2 | All radiographic responders: *Ruminococcaceae* OTU185 spp., CAG-352 OTU217 spp., *Prevotella* OTU264 spp., *Dialister* OTU18 spp., *Lachnospiraceae* OTU77 spp., *Ruminiclostridium* OTU216 spp., *Lachnospira* OTU159 spp., *Ruminococcus* OTU222 spp., *Ruminiclostridium* OTU196 spp., *Parabacteroides* OTU295 spp.<br>Esophageal carcinoma ($N = 14$): *Prevotella* _9 OTU261 spp., *Dialister* OTU18 spp., *Lachnospiraceae* OTU97 spp., Ruminococcus_2 OTU222 spp., *Bacteroides* OTU266 spp., *Parasutterella* OTU207 spp., *Phascolarctobacterium* OTU1 spp., *Bacteroides* OTU277 spp., *Lachnospiraceae* OTU105 spp., *Lachnospiraceae* OTU128 spp.<br>Gastric cancer ($N = 23$): *Prevotella_9* OTU263 spp., *Bifidobacterium* OTU202 spp., *Prevotella* _2 OTU264 spp., *Lachnospira* OTU156 spp., *Bacteroides* OTU274 spp., *Ruminococcaceae* OTU190 spp., *Agathobacter* OTU79 spp., *Bacteroides* OTU275 spp.<br>Colorectal carcinoma ($N = 19$): *Lachnoclostridium* OTU104 spp., *Parabacteroides* OTU294 spp., *Parabacteroides* OTU293 spp., *Lachnospira* OTU155 spp., *Ruminococcaceae* OTU187 spp., *Flavonifractor* OTU197 spp., *Dialister* OTU18 spp., *Ruminococcaceae* OTU187 spp., *Lachnospiraceae* OTU77 spp., *Lachnospiraceae* OTU103 spp. | Diversity metrics were not associated with outcome. |

Abbreviation: MSS, microsatellite stable.

[a]In Hakozaki and colleagues, while stable disease (SD) was observed in 17 patients and patients with SD ≥ 6 months were counted as R, this number was not reported.

observed in PD-1–treated melanoma apply to other cancers treated with other ICIs and/or ICI combinations will need to be evaluated. Developing novel analytic methods to better explore the individual-specific microbial diversity will aid these endeavors (58–60).

## Potential mechanism(s) to explain the impact of gut microbiota on cancer immunotherapy

The precise mechanisms underlying how gut microbiota influence cancer immunotherapy are poorly understood, but research has converged upon three themes (see below; and **Fig.** 1): bacteria or bacterial components that directly stimulate antitumor T-cell responses, molecular mimicry between shared bacterial and tumoral epitopes, and bacterial metabolites that shape antitumor immunity.

Some gut bacteria can elicit defined antigen-specific T-cell responses, including *Helicobacter* spp. [RORγt$^{+}$ FOXP3$^{+}$ inducible T-regulatory cells (iTreg)] and *A. muciniphila* (IgG1 antibodies and Tfh cells; refs. 61–64). Others exert immunostimulatory properties either directly or after being sensed by DCs in gut-associated lymphoid tissue (GALT), spleen, and/or tumor draining lymph nodes. These include: *B. thetaiotaomicron* or *B. fragilis*, which enhance the efficacy of CTLA-4 treatment (22), and *E. hirae* and *B. intestihominis*, which directly enhance the intratumoral CD8/Treg ratio and IFNγ-producing γδ T cells, respectively, following cyclophosphamide treatment (14). Others include *B. rodentium,* which stimulates antitumor responses in *Rnf5*$^{-/-}$ mice in a My8DD/TLR-mediated fashion (65), and *Bifidobacterium* spp., which sensitizes mice to anti-CD47 immunotherapy in a STING-and IFN-dependent fashion (21). Separately, bacterial flagellin directly interacts with TLR5; in cancer, bacterial flagellin derived from *E. gallinarum* and *S. typhimurium* demonstrate immunostimulatory potential (66, 67).

Molecular mimicry between pathogens and tumor antigens can also elicit cross-reactive T cells via antigenic mimicry (**Fig.** 1). Preclinically, both *Bifidobacterium breve* and the *E. hirae* bacteriophage elicited commensal-specific T cells that cross-reacted with candidate neoantigens (19, 68). In humans, long-term survival in pancreatic cancer was associated with the development of highly immunogenic neoantigens with predicted cross-reactivity to microbial epitopes (69). *Fusobacterium nucleatum* is associated with colorectal cancer and promotes colonic tumor formation in preclinical models; this bacterium interacts with the inhibitory T-cell receptor TIGIT through FAP2 and can directly suppress antitumor immunity and inhibit tumor killing by natural killer (NK) cells (70, 71). Collectively, these data suggest that certain commensals may influence adaptive and/or innate responses to cancer by modulating inhibitory checkpoint pathways. Finally, human

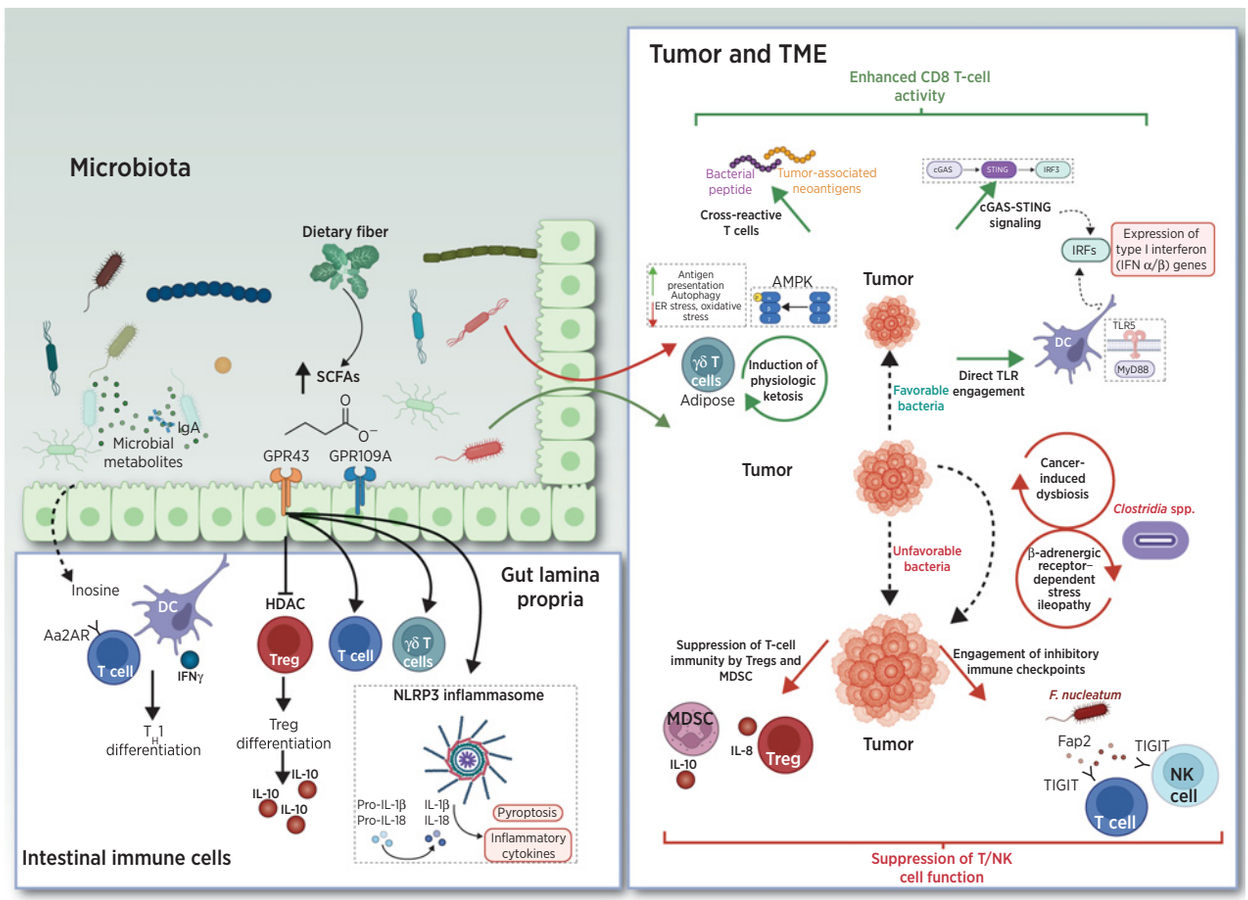


**Figure 1.**
Mechanisms for the effects of bacteria or bacterial metabolites on antitumor immune responses. MDSC, myeloid-derived suppressor cell; TME, tumor microenvironment.

melanoma expresses a repertoire of human leukocyte antigen class I (HLA-I) and class II (HLA-II) restricted peptides derived from intratumoral bacteria that can be presented by antigen-presenting cells and elicit T-cell responses (72).

Metabolites synthesized *de novo* by gut microbiota or produced by the host and biochemically modified by gut bacteria can exert immunomodulatory effects (**Fig.** 1). Endogenous nucleosides adenosine and inosine exert a wide range of anti-inflammatory and immunomodulatory effects *in vivo* through adenosine receptors (AR). Pharmacologic inhibition of adenosine signaling—either through direct $A_{2A}R$ inhibition or inhibition of CD38, CD39, or CD73—enhances antitumor immunity in multiple preclinical models and is a focus of drug development (73, 74). Surprisingly, supplementation of inosine, which is formed by adenosine catabolism, was recently reported to enhance efficacy of anti–PD-1 therapy and cell therapy but only in a low-glucose environment (75). In multiple mouse tumor models, key gut microbiota—specifically *A. muciniphila* and *B. pseudolongum*—produce inosine and enhance the effects of anti–CTLA-4 therapy (76).

Major classes of microbial-derived molecules such as short-chain fatty acids (SCFA) and secondary bile acids (BA) are immunomodulatory (**Fig** 1). Commensal bacteria convert primary to secondary BAs, which promotes hepatic $CXCR6^+$ NKT cells that inhibit liver-selective metastases in preclinical models of primary and metastatic liver cancer (77). SCFAs such as acetate, propionate, and butyrate are the byproduct of bacterial fermentation of dietary fiber in the colon (78). These products bind to specific SCFA-sensing G-protein–coupled receptors (GPCR; GPR41, GPR43, GPCR109A) and regulate histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity. SCFAs modulate intestinal immune homeostasis, affecting Tregs, effector T cells, and γδ T cells (79–85). Preclinically, the effects of SCFA supplementation in the context of cancer immunotherapy is controversial. Both favorable or unfavorable effects may occur (86–88). In two separate studies of anti–PD-1–treated patients with cancer, higher levels of SCFAs were associated with favorable response (89, 90). However, given the relatively small number of patients evaluated in both studies and the context-dependent cues governing SCFA production in humans that were not independently evaluated, the role of SCFA supplementation upon ICI efficacy remains unclear.

Ketone bodies are a vital alternative metabolic fuel source for all domains of life. Ketogenic diets (KD) alter the composition of gut microbiota in mice (91, 92). Further, systemic ketosis increases the abundance of bile-tolerant members of the Bacteroidetes phylum (*Alistipes* spp., *Bilophila* spp., and *Bacteroides* spp.) and decreases the levels of butyrate-producing members of the Firmicutes phylum (*Roseburia* spp., *Eubacterium rectale*, and *Ruminococcus bromii*) in humans (93, 94). KDs improve tumor antigen-specific innate and adaptive immune responses in multiple preclinical models (95, 96), possibly related to 3-hydroxybutyrate-mediated increases in *Eisenbergiella massiliensis* (97) or direct AMP-activated protein kinase (AMPK) activation and consequent enhancement of antigen presentation (97). KDs also appear to expand protective adipose-resident γδ T cells (98). However, it is unclear whether the observed immunological effects are secondary to gut microbiome-induced changes in the intestinal immune environment or due to circulating ketone bodies that may directly affect immune-cell function (98, 99).

In McCulloch and colleagues, noninvasive exfoliated transcriptome (exfoliome) analysis implicated LPS-producing Gram-negative bacteria enriched for key bacterial genes including α-l-fucosidase, α-galactosidase, and glycosyltransferases in mediating nonresponse to PD-1 (50). These genes are linked with mucus degradation and LPS synthesis, respectively, suggesting potential mechanisms of action used by the human gut microbiota to modulate host immunity and outcome upon PD-1 ICI therapy (100–102).

## Preclinical and human data implicating gut microbiome composition in the development of adverse events to anticancer agents

In addition to their putative antitumor effects, commensal organisms are strongly implicated in mediating toxicity to a spectrum of anticancer agents, including ICIs. Commensal microbiota mediate methotrexate-induced gut toxicity controlled by *ABCB1/MDR1*-encoded p-glycoprotein drug efflux pumps in $CD11b^+$ intestinal myeloid cells in a TLR2-dependent fashion (103). Irinotecan (CPT-11)-related diarrhea is mediated by metabolite SN-38, in turn mediated by microbial β-glucuronidases (GUS). Inhibitors of GUS enhance irinotecan efficacy by blocking the irinotecan-induced bloom of *Enterobacteriaceae* spp. (104). Streptomycin reduces the severity of irinotecan-mediated delayed-onset diarrhea by reducing ileum and colonic tissue exposure to irinotecan and related metabolites (105). Gemcitabine (dFdC) is less efficacious in the presence of certain bacteria, e.g., *Gammaproteobacteria* spp., that have increased expression of a long isoform of bacterial cytidine deaminase ($CDD_L$). This $CDD_L$ expression increases metabolic degradation of gemcitabine into difluorodeoxyuridine (dFdU; ref. 106). Thus, antibiotic use is associated with increased gemcitabine efficacy in preclinical models of pancreatic cancer (106) and improved survival of humans with pancreatic cancer (107).Tyrosine kinase inhibitor (TKI) therapy-related diarrhea is associated with higher levels of *Bacteroides* spp. and lower levels of *Prevotella* spp. in patients with RCC (108).

During PD-1 and/or CTLA-4 ICI treatments, species belonging to Bacteroidetes, Clostridia, and Proteobacteria phyla have been linked to increased incidence and severity of immune-related adverse events (irAE; refs. 35, 109, 110). Of these events, ICI-related colitis has been most closely linked with commensal microbiota, specifically members of the Firmicutes phyla (*F. prausnitzi*, *G. formicilis*, *Ruminococcaceae* spp.), during anti–CTLA-4 ICI therapy (35, 109). In addition, *B. intestinalis* (phylum Bacteroidetes) was linked to colitis in patients treated with anti–PD-1 and anti–CTLA-4 combination therapy (110).

McCulloch and colleagues identified two microbial signatures associated with specific irAE profiles and divergent clinical outcomes (58). One signature exhibited increased abundance of members of the Lachnospiraceae family and was associated with favorable response to anti–PD-1. The other was dominated by *Streptococcus* spp. and associated with shorter PFS and a high frequency of distinct irAEs, particularly arthritis (58). Increased abundance of *Streptococcus* was associated with proton pump inhibitor (PPI) use. Thus, it is tempting to speculate that PPIs increase the survival rate of oral bacteria during gastric passage (i.e., oralization), inducing a shift in gut microbiome composition. Altogether, these findings reconcile the discordant published results linking irAEs and clinical response to anti–PD-1 (58).

Single-cell analyses of intestinal luminal samples from ICI-treated patients who developed colitis identified transcriptionally-distinct T cells (characterized by *IFNG*, *GBP5*, *HLA-DR*, and *CD74* expression) and myeloid cells (characterized by *TNF*, *IL1B*, and *OSM* expression) that drove the emergence of $CD8^+$ T-effector cells (111). Similarly, increased numbers of IFN-expressing $CD8^+$ effector T cells and IL1β-expressing monocytes have been related to ICI-related pneumonitis (112). Thus, it is unsurprising that immunosuppressive therapies,

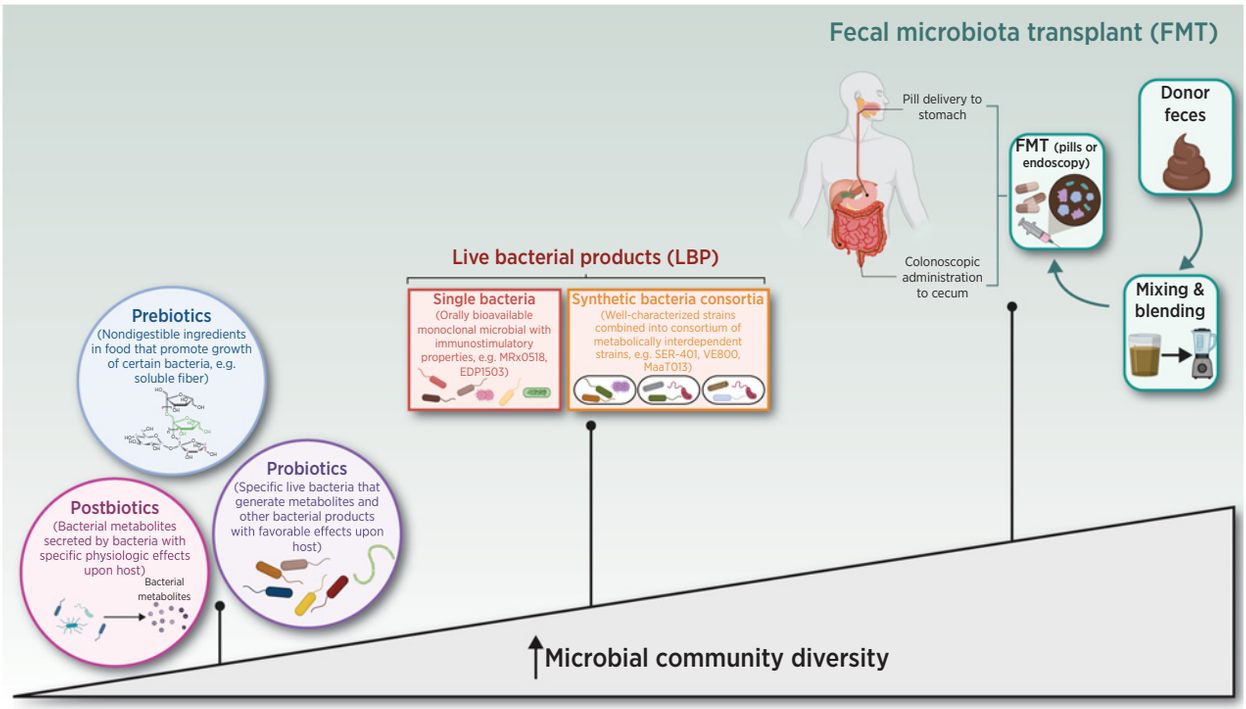


**Figure 2.**
Spectrum of microbiome-specific interventions for the treatment of cancer.

including corticosteroids and TNFα inhibitors, are effective in treating ICI-related colitis and pneumonitis. More recently, healthy donor-derived FMT was found to efficiently treat biologic (TNFα and/or integrin $\alpha_4\beta_7$ inhibitor) refractory ICI colitis, supporting that pathobionts can mediate the development of this irAE (113, 114). These observations suggest pathobionts influence the development of irAEs in epithelial organs with a local microbiota and large populations of tissue-resident T cells via a TNF/CXCL8/IL1β and NLRP3 inflammasome-mediated program. Whether this holds true for other irAEs in epithelial organs besides the colon, e.g., the skin and/or lung, remains unknown.

## Microbiome-based therapy of cancer

Most therapeutic interventions aimed at targeting the microbiome in cancer have focused on the gut (rather than the skin or the pulmonary) microbiome; these approaches are summarized in **Fig. 2** and **Table 2**. Patients with Lugano stage I or II of *Helicobacter pylori* (*H. pylori*)-associated gastric marginal zone lymphoma (MZL) are typically treated with *H. pylori*–directed triple or quadruple antibiotic therapy (115). Outside of this, there is little therapeutic use of antibiotics in cancer. Preclinical studies suggest that antibiotic administration improves outcomes in models of primary/metastatic lung, colon, and pancreatic cancer by facilitating a more immunogenic tumor microenvironment (116–119), but accumulating evidence from patients with solid organ cancer tumors treated with ICI therapy indicates that systemic antibiotic therapy associates with reduced bacterial diversity, diminished ICI efficacy, and poorer survival in multiple cancers (29, 31–34). Similarly, the use of antibiotics is associated with leukemic progression in genetically predisposed hosts (120, 121). Therapeutic and/or prophylactic use of broad-spectrum antibiotics in patients with hematologic malignancies during allogeneic hematopoietic stem cell transplantation (allo-HSCT) is associated with profound changes in intestinal microbiota composition, particularly with loss of organisms belonging to the Clostridia class and Bacteroidetes phylum (122–126). These changes are associated with lower bacterial diversity, increased risk of systemic infection with multi-drug resistant bacteria, higher rates of graft-versus-host disease (GVHD), and GVHD-related mortality (122–126).

The effects of dietary intake upon cancer incidence, progression, and therapeutic outcomes are broad and all-encompassing. However, compelling epidemiologic associations are undermined by inconsistent dietary data collection across studies precluding deeper mechanistic insights, as reviewed in detail elsewhere (127). Across studies, the intake of insoluble dietary fiber (DF), which is not digested but instead fermented into SCFAs by gut bacteria, is associated with increased production of $CD103^+$DCs, enhanced differentiation of activated $CD8^+$T cells into effector cells with memory phenotype (128, 129), and improved outcomes to anti–PD-1 ICI therapy in patients with cancer (130, 131). Specifically, increased intake DF (defined as 20 g/day) was associated with significantly improved PFS in a retrospective analysis of ICI-treated patients with melanoma. The most pronounced benefit was observed in patients with sufficient DF intake and no probiotic use (131).

Multiple studies are evaluating prebiotics (molecules that promote growth of beneficial microbes), probiotics (live microorganisms with putative benefit), and/or postbiotics (microbial-derived molecules) during cancer treatment. Prebiotics, including insoluble DF, demonstrate promise in preclinical models of melanoma and colon cancer, and several clinical trials are evaluating this in patients

**Table 2.** List of clinical trials evaluating microbiome therapeutics in cancer.

| Sponsor and trial information | Nature of product | Clinical trial information | Dose | Antibiotic conditioning | Enrollment status | ORR/DCR | TRAE |
|---|---|---|---|---|---|---|---|
| **FMT** | | | | | | | |
| Baruch et al. (142) | Donor (melanoma PD-1 responder) | PD-1 primary and secondary refractory melanoma | Colonoscopic FMT (day 0), then oral FMT capsules (day 1 and day 12), repeated every 2 weeks along with nivolumab. Nivolumab 240 mg every 2 weeks. | Yes | Not active | 30% | No moderate-to-severe irAEs |
| Davar et al. (51) | Donor (melanoma PD-1 responder) | PD-1 secondary refractory melanoma | Single responder-derived FMT (day 1). Pembrolizumab 200 mg every 3 weeks. | No | Not active | 20%/40% | 3 grade 3 irAEs (2 instances fatigue, 1 peripheral motor neuropathy) |
| NCT04729322 | Donor (dMMR–PD-1 responder) | dMMR patients following progression on PD-1 | Cycle 1: FMT induction via colonoscopy (day 5), then capsules on days 1, 8, 15. Cycles 2+: FMT capsules on day 1 every 3 weeks. | Metronidazole, vancomycin, neomycin | Active, enrolling | Not reported | Not reported |
| NCT04130763 | Healthy donors with microbiome profiles similar to PD-1 responders | GI cancers following progression on PD-1 | Cycle 1: FMT capsules for 1 week as induction. Cycles 2: FMT capsule maintenance. | None | Active, enrolling | Not reported | Not reported |
| MiMic (NCT03772899) | Healthy donor | PD-1 naïve metastatic melanoma in combination with nivolumab | FMT induction preimmunotherapy. FMT maintenance with nivolumab or pembrolizumab. | None | Active, enrolling | Not reported | Not reported |
| **Complete consortia products** | | | | | | | |
| MCGRAW (NCT03817125, Seres) | Orally bioavailable encapsulated consortia of commensal bacteria | PD-1 naïve metastatic melanoma in combination with nivolumab vs. placebo<br>PD-1 refractory melanoma in combination with nivolumab | SER-401 capsule once daily for 7 days (lead-in), then daily. Nivolumab per label. | Vancomycin, 5 days | Active, not enrolling | Not reported | Not reported |
| PICASSO (NCT03772899, MaaT Pharma) | Healthy donor–derived full-spectrum microbiome therapeutic (MaaT033) | PD-1 naïve metastatic melanoma | MaaT033 every 3 weeks (weeks 0–9), then every 12 weeks (weeks 15–23); total 7 infusions. | None | Active, enrolling | Not reported | Not reported |
| Synthetic bacterial consortia | | | | | | | |
| VE800 (Vedanta) | Orally bioavailable LBP containing 11 distinct nonpathogenic, nontoxigenic, commensal bacterial strains | Select histologies following failure of standard therapy in combination with pembrolizumab (NCT04208958):<br>Melanoma<br>MSS CRC<br>Gastric and GE cancer | VE800 5 capsules loading, then 2 capsules daily.<br>Nivolumab per label. | Vancomycin, 5 days | Active, not enrolling | Not reported | Not reported |
| **Monoclonal microbial candidates** | | | | | | | |
| Miyarisan Pharmaceutical | Orally bioavailable bifidogenic *Clostridium butyricum* strain MIYAIRI588 (CBM588) | Advanced RCC in combination with nivolumab and ipilimumab (NCT03829111) (143) | CBM588 890 mg twice daily. | None | Completed | 58% (nivo/ipi + CBM588) vs. 20% (nivo/ipi) | Grade 3/4 AEs: 52% (nivo/ipi + CBM588) vs. 50% (nivo/ipi) |
| | | Advanced RCC in combination with nivolumab and cabozantinib (NCT05122546) | | None | Active, enrolling | Not reported | Not reported |
| Evelo | Orally bioavailable monoclonal microbial derived from single clone of *Bifidobacterium* spp. | PD-1 refractory melanoma in combination with pembrolizumab (NCT03595683) | EDP1503 capsules twice daily for 14 days (lead-in), then twice daily; each capsule contains ≥ $7.5 \times 10^{10}$ CFUs. Pembrolizumab per label. | None | Suspended | Not reported | Not reported |
| | | Multiple histologies following failure of standard therapy in combination with pembrolizumab (NCT03775850):<br>Cohort A: MSS CRC<br>Cohort B: TNBC<br>Cohort C: NSCLC, bladder cancer; GE cancer, any microsatellite unstable, or RCC | | None | Active, not enrolling | Not reported | Not reported |

*(Continued on the following page)*

**Table 2.** List of clinical trials evaluating microbiome therapeutics in cancer. (Cont'd)

| Sponsor and trial information | Nature of product | Clinical trial information | Dose | Antibiotic conditioning | Enrollment status | ORR/DCR | TRAE |
|---|---|---|---|---|---|---|---|
| NCT03637803 (4d Pharma) | Orally bioavailable LBP containing *Enterococcus gallinarum* flagellin, which has TLR5 stimulatory properties | Select histologies following failure of standard therapy in combination with pembrolizumab (NCT03775850):<br>RCC<br>NSCLC | MRx0518 capsules twice daily. Pembrolizumab per label. | None | Active, not enrolling | Not reported | Not reported |
| **Oncomimics** | | | | | | | |
| ROSALIE, NCT04116658 (Enterome) | EO2401: multipeptide (3) vaccine containing peptides (oncomimic), which are strongly homologous but not identical to key tumor antigens in select tumors | Glioblastoma following failure of standard therapy in 3 cohorts:<br>Cohort 1: EO2041 monotherapy, followed by sequential EO2041 and nivolumab<br>Cohort 2: Concurrent EO2041 and nivolumab<br>Cohort 3: EO2041 and nivolumab and bevacizumab | EO2401 multiple doses. Nivolumab and bevacizumab per label. | None | Active, not enrolling | Not reported | Not reported |
| **Prebiotics, postbiotics, probiotics, and dietary interventions** | | | | | | | |
| NCT05220124 (Tianjin Medical University Second Hospital, Tianjin, China) | Live combined (*Bifidobacterium* spp., *Lactobacillus* spp., and *Enterococcus* spp.) probiotic capsules | Prospective phase IV randomized trial of immunotherapy ± live combined probiotic capsules in platinum-ineligible metastatic urothelial cancer | Live combined probiotic capsules 420 mg twice daily for 3–4 cycles. | None | Active, enrolling | Not reported | Not reported |
| NCT04699721 (Xiangya Hospital of Central South University, Hunan, China) | Unnamed probiotic | Phase I trial of nivolumab with carboplatin and paclitaxel and probiotic in resectable NSCLC | Not stated | None | Active, enrolling | Not reported | Not reported |
| EDEN, NCT04866810 (NIH) | Dietary and exercise intervention | Randomized phase II study of anti–PD-L1 immunotherapy ± dietary and exercise intervention in cancer patients | Plant-based, high-fiber diet and exercise (150 minutes moderate-intensity, 75 minutes high-intensity weekly) | None | Active, enrolling | Not reported | Not reported |
| DIET (NCT04645680) | Isocaloric high-fiber diet | Randomized, double-blind phase II study evaluating high-fiber diet in patients with resectable stage III/IV melanoma following definitive surgery | Isocaloric high-fiber diet (Arm 1) vs. isocaloric control diet (Arm 2) | None | Active, enrolling | Not reported | Not reported |
| NCT03700437 | Intermittent fasting diet | Nonrandomized pilot phase II study of intermittent fasting diet in patients with stage III NSCLC following definitive chemoimmunotherapy (CIT) | Chemolieve®, a plant-based FMD that provides ∼300 calories/fasting day | None | Active, enrolling | Not reported | Not reported |
| CETOREIN (NCT04316520) | Ketogenic diet | Nonrandomized pilot phase II study of ketogenic diet in patients with advanced RCC receiving immunotherapy | Ketogenic diet | None | Active, enrolling | Not reported | Not reported |

Abbreviations: CFU, colony-forming units; dMMR, deficient mismatch repair; FMD, fasting mimicking diet; GE, gastroesophageal; LBP, live bacterial product; NHL, non-Hodgkins lymphoma; nivo/ipi, nivolumab/ipilimumab; NSCLC, non–small cell lung cancer; MSS CRC, microsatellite stable colorectal carcinoma; TNBC, triple-negative breast cancer; RCC, renal cell carcinoma.

with melanoma (DIET - NCT04645680, EDEN - NCT04866810), NSCLC (NCT03700437), and RCC (NCT04316520; refs. 132, 133). Interestingly, one preclinical study has recently shown that natural polyphenol castalgin exerts antitumor activity in multiple mouse tumor models to overcome resistance to anti–PD-1 by modulating gut microbiota (134). There is currently a lack of experimental evidence for the use of postbiotics, at least in the context of cancer. A prospective clinical trial compared high-fiber (20 g/day) to high fermented food (6 servings/day) diets in healthy adults and found the latter was associated with increased microbial diversity and reduced systemic inflammation during the 10-week dietary intervention (135). These data suggest that, while the human microbiome is relatively recalcitrant to rapid diet-induced remodeling, specific changes introduced gradually may be leveraged to improve human health.In the context of metabolic disease, an inpatient crossover study compared a ketogenic with an isocaloric control diet in adult men without diabetes but with overweight or obesity. The KD increased Bacteroidetes and Proteobacteria and reduced Actinobacteria and Firmicutes, resulting in decreased intestinal Th17 cell levels (99). KDs in patients with cancer are feasibile and efficient in inducing ketosis (136–140), but the effects of KDs on anticancer treatments have been variable, with favorable effects in patients with breast cancer undergoing neoadjuvant chemotherapy (137) but no effects on reirradiated malignant glioma (138).

The efficacy of FMT in treating ICI-resistant cancers was demonstrated in two clinical trials (51, 141). Both trials evaluated the response of patients with PD-1 refractory melanoma [either primary (51) or secondary (141) to anti–PD-1 therapy combined with FMT obtained from individual long-term responders (R; R-FMT)]. The studies differed in the number of long-term responder donors (2 vs. 8), nature of host microbiota depletion (antibiotics vs. polyethylene glycol-electrolyte laxative), number of patients treated (10 vs. 16), and number of R-FMT administered (multiple vs. single). Both studies demonstrated that R-FMT effectively shifted the microbiome composition toward taxa favoring anti–PD-1 efficacy and reported that R-FMT was associated with increased intratumoral and peripheral antitumor immunity. Davar and colleagues found donor engraftment in 10 of 15 recipients that was sufficient to induce response in 6 patients. In contrast, complete donor engraftment was not required to ameliorate refractory *Clostridium difficile*colitis (rCDI; ref. 142). A recent study indicated that antibiotic duration and FMT dosing frequency influences engraftment in non–germ-free mice (143), suggesting that the intestinal dysbiosis observed in PD-1–resistant melanoma may be more "refractory" than rCDI and thus require more extended dosing.

Rationally designed bacterial consortia, monoclonal microbial candidates and bacterial peptides (oncomimics) are being evaluated in combination with ICIs across multiple indications (**Table 2**). Tanoue and colleagues used a preclinical system previously used to characterize Treg-inducing bacteria from healthy human donor stool to isolate a consortium of 11 bacterial strains. These induced IFNγ-producing $CD8^+$ T cells in the lamina propria and systemically in a $CD103^+$DC- and MHC class I–dependent manner (144). This and other consortia (MET-4, NuBiyota) are being studied in clinical trials in combination with anti–PD-1 in advanced cancer. Other strains being investigated include *E. gallinarum* (MRx0518), *B. animalis lactis* (EDP1503), and *C. butyricum* (CBM588). A phase II trial evaluating the butyrate-producing bacterium *Clostridium butyricum* (CBM588) in combination with nivolumab/ipilimumab reported that the addition of CBM588 increased response rates in patients with advanced intermediate- and poor- risk RCC (145). Patients treated with CMB588 had a higher response rate than controls (58% vs. 20%), but, surprisingly, the control arm performed poorly when compared with historical controls (20% vs. 42% in CheckMate 214) and the CBM588-treated patients did not exhibit a pharmacodynamic signal indicating increased bifidogenic bacteria. Synthetic biology—or the specific engineering of bacteria for a defined purpose—offers another therapeutic approach. This approach was used, preclinically, for the controlled production and intratumoral release of nanobodies targeting PD-1/CTLA-4 (146) and CD47 (147) within the tumor microenvironment.

## Conclusion

The gut microbiome plays an established and critical role in regulating antitumor immunity and responses to ICI in patients with cancer; mounting evidence suggests the intratumoral microbiome may do the same. Preclinical studies identified some mechanisms underlying the immune and antitumor effects of specific bacteria, but more studies are needed to elucidate how gut bacteria augment or impede antitumor immunity in humans. Uncovering these mechanisms will require scaled evaluations of gut microbiota in large and deeply phenotyped cancer cohorts integrating patient-level information (outcome, irAE incidence) and the many variables that may influence gut microbiome composition both at the patient (diet, medication use) and cohort (geography, ethnicity) levels. Uniform sample collection and processing methods are also necessary.

Clinical trials evaluating FMT in refractory melanoma indicate this new modality may be used to modulate gut microbiota in cancer. Additional well-designed interventional clinical trials in patients with melanoma are required to confirm these results. Others are needed to generate preliminary data for additional cancers where ICI efficacy has been linked to gut microbiota (NSCLC, RCC, etc.). It remains to be seen whether defined consortia can produce the same benefits as FMT-based therapies. Finally, mechanistic studies in mouse tumor models will be needed to help validate hypotheses raised by studies in patients with cancer.

### Authors' Disclosures

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