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Role of Microbiome in Oropharyngeal Squamous Cell Carcinoma (ROMA)

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Role Of Microbiome in Oropharyngeal Squamous Cell Carcinoma (ROMA)

Doctoral thesis dissertation presented by Marc Oliva to apply for the degree of doctor at the University of Barcelona

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UHN Princess Margaret Cancer Centre

ICO Institut Català d'Oncologia

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Abbreviations and acronyms

AE: Adverse events

ASV: Amplicon sequence variant

BA: Bile acids

CRT: Chemoradiotherapy

CTLA-4: T-lymphocyte-associated antigen 4

DNA: Deoxyribonucleic acid

FMT: Fecal Microbial Transplantation

HPV: Human Papillomavirus

ICI: Immune checkpoint inhibitors

LA: locally-advanced

MAMP: microbial-associated molecular pattern

MET: Microbial Ecosystem Therapeutics

OPSCC: Oropharyngeal squamous cell carcinoma

ORR: Overall response rate

OS: Overall survival

OTU: Operational Taxonomic Unit

RFS: Recurrence-free survival

RNA: Ribonucleic acid

PD-(L)1: Programmed cell-death protein (ligand)-1

PFS: Progression-free survival

RA: Relative abundance

ROMA: Role of microbiome as a biomarker in OPSCC

SCFA: short-chain fatty acids

List of articles in the thesis

Thesis in compendium of publications format.

The thesis consists of 5 objectives and a total of 2 articles. The publications included in this thesis are:

1. **Marc Oliva**, Pierre H. H. Schneeberger, Victor Rey, Matthew Cho, Rachel Taylor, Aaron R. Hansen, Kirsty Taylor, Ali Hosni, Andrew Bayley, Andrew J. Hope, Scott V. Bratman, Jolie Ringash, Simron Singh, Ilan Weinreb, Bayardo Perez-Ordoñez, Douglas Chepeha, John Waldron, Wei Xu, David Guttman, Lillian L. Siu, Bryan Coburn and Anna Spreafico. *Transitions in oral and gut microbiome of HPV+ oropharyngeal squamous cell carcinoma following definitive chemoradiotherapy (ROMA LA-OPSCC study).* British Journal of Cancer. 2021 Apr;124(9):1543-1551.
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   - JCR: Q1

2. **Marc Oliva**, Alya Heirali, Geoffrey Watson, Ashley M. Rooney, Kyla Cochrane, Sarah Jennings, Rachel Taylor, Minge Xu, Ali Hosni, Andrew Hope, Scott V. Bratman, Douglas Chepeha, Ilan Weinreb, Bayardo Perez-Ordonez, Ricard Mesia Nin, John Waldron, Wei Xu, Aaron Hansen, Lillian L Siu, Bryan Coburn and Anna Spreafico. *Prospective manipulation of the gut microbiome with Microbial Ecosystem Therapeutic 4 (MET-4) in HPV-related locoregionally-advanced oropharyngeal cancer squamous cell carcinoma (LA-OPSCC) undergoing primary chemoradiation: ROMA2 study.* British Journal of Cancer. 2024 May 7. Online ahead of print.
   - Impact factor (2024): 8.8
   - JCR: Q1

Thesis Summary/Resum de la tesi

Títol: Paper del microbioma com a biomarcador en el carcinoma escatós d'orofaringe (ROMA)

Introducció

El microbioma associat a tumor sembla tenir un paper rellevant a l'oncogènesi, la progressió i la resposta a tractaments de múltiples càncers, incloent-hi els carcinomes escatosos de cap i coll. A més, diversos estudis indiquen que el microbioma intestinal influeix en la resposta immune antitumoral i prediu la resposta a immunoteràpia. Per tant, les estratègies de modulació del microbioma per optimitzar-ne l'eficàcia són un àrea de gran interès.

Hipòtesis

1. Els pacients amb carcinoma escatós d'orofaringe localment avançat (LA-OPSCC) relacionat amb el Virus del Papil.loma Humà (VPH) tenen una composició específica del microbioma oral i intestinal abans de la quimioradioteràpia radical.
2. La quimioradioteràpia té un impacte en la composició i diversitat del microbioma oral e intestinal.
3. La modulació del microbioma intestinal amb MET-4 (una barreja d'espècies bacterianes relacionades amb la resposta immune) és factible i segura en el context de la quimioradioteràpia radical.
4. L'administració de MET-4 comporta un augment qualitatiu i quantitatiu de les especies bacterianes incloses en el mateix MET-4 en mostres de femta.
5. Els canvis produïts per MET-4 en la composició del microbioma intestinal estan associats a canvis metabolòmics intestinals i plasmàtics.

Objectius

1. Caracteritzar la composició i diversitat del microbioma oral i intestinal a la saliva, frotis tumoral i femta en pacients amb LA-OPSCC VPH-relacionat abans d'iniciar el tractament.
2. Avaluar l'impacte de la quimioradioteràpia radical sobre la composició (abundància relativa i diversitat) del microbioma oral e intestinal.

3. Avaluar la factibilitat i seguretat de la modulació del microbioma intestinal mitjançant l'administració oral de MET-4 de forma concurrent a la quimioradioterapia en pacients amb LA-OPSCC HPV-relacionat.
4. Avaluar la presencia qualitativa i quantitativa de les espècies bacterianes incloses en MET-4 en femta després de la seva administració.
5. Avaluar els canvis en la composició metabolòmica en femta i plasma posteriors a l'administració de MET-4.

Material/Mètodes

Per als objectius 1 i 2, es realitzà un estudi prospectiu observacional en una cohort de pacients amb diagnòstic de LA-OPSCC VPH-relacionat candidats a quimioradioteràpia radical (ROMA LA-OPSCC-01). Es recolliren mostra de saliva, frotis tumoral i mostra de femta de cada pacient abans i després del tractament. Es realitzà seqüenciació de la subunitat 16S del ARN ribosomal en cada una de les mostres per determinar la composició bacteriana del microbioma oral (saliva), associat a tumor (frotis tumoral) i intestinal (femta). Mitjançant anàlisi bioinformàtic es determinà la composició bacteriana en termes d'abundància relativa i la diversitat (alpha/beta) de les mostres. Es realitzà anàlisi bioestadístic descriptiu i inferencial de les dades obtingudes.

Per als objectius 3-5, es dissenyà un assaig clínic d'un sol braç per a pacients amb LA-OPSCC VPH-relacionat candidats a quimioradioteràpia radical (ROMA LA-OPSCC-02). Durant les primeres 3 setmanes del tractament els pacients rebien addicionalment MET-4 per via oral (una barreja de bacteris favorables a la resposta immune derivats de femta humana i cultivats in vitro). Es recolliren frotis tumoral, femta, i plasma abans de començar el tractament, un cop finalitzada l'administració de MET-4 (setmana 4), al finalitzar el tractament amb quimioradioteràpia i als dos mesos de seguiment. Es realitzà seqüenciació de la subunitat 16S del ARN ribosomal en cada una de les mostres per determinar la composició bacteriana del microbioma associat a tumor (frotis tumoral) i intestinal (femta). Mitjançant cromatografia líquida/espectrometria de masses en tàndem es determinaren els perfils metabolòmics en plasma i femta.

Principals resultats

A l'estudi ROMA LA-OPSCC-01, es van incloure 22 pacients i es van analitzar 132 mostres. La composició del microbioma analitzat a saliva i frotis tumoral va ser similar (R2 =0,006; p=0,827). La composició bacteriana del microbioma en frotis tumoral fou significativament diferent segons l'estadiatge tumoral, amb un augment de l'abundància relativa de Fusobacterium nucleatum en pacients amb estadi III respecte I-II (p<0, 05). La quimioradioteràpia va impactar de forma significativa en la diversitat i composició del microbioma associat a tumor, amb disminució del nombre de soques i un augment de l'abundància relativa de bactèries associades a flora intestinal (p<0,05), mentre que no va tenir cap efecte en la composició del microbioma intestinal. Aquestes troballes es van mantenir significatives quan es van ajustar els canvis en base a l'estadi tumoral i l'ús d'antibiòtics.

A l'estudi ROMA LA-OPSCC-02, un total de 30 pacients van ser inclosos i 29 van rebre almenys una dosi de MET-4 i per tant, foren avaluables per l'objectiu de tolerància i seguretat. Es van produir esdeveniments adversos relacionats amb el fàrmac en 13/29 pacients: tots de caràcter lleu (graus 1-2, CTCAEv5.1) excepte un event grau 3 (diarrea). MET-4 es va suspendre abans de completar les 3 setmanes planificades en 7/29 pacients a causa de la toxicitat induïda per la quimioradioteràpia, i en 1/29 a causa del MET-4 (diarrea grau 3 que es va autolimitar). Vint pacients van ser avaluables per als objectius relacionats amb la composició del microbioma i dels metabolomes fecals i plasmàtic. No hi va haver augment en el nombre absolut ni l'abundància relativa de les soques bacterianes incloses a MET-4 després de la seva administració, però si van augmentar de forma quasi significativa en pacients amb malaltia tumoral estadi III (p=0,06). La RA MET-4 va ser més alta en els pacients amb estadi III enfront dels estadis I-II a la setmana 4 post-administració (p=0,03) i 2 mesos d'haver finalitzat el tractament (p=0,01). Aquest augment es va correlacionar amb canvis en la composició metabolòmica fecal i plasmàtica.

Conclusions

- El pacients amb LA-OPSCC VPH-relacionat tenen un microbioma oral e intestinal específic que es relaciona amb l'estadi tumoral i pot variar per l'efecte de la quimioradioteràpia.

- En particular, els pacients amb malaltia d'alt risc (estadi III) presenten un microbioma oral e intestinal potencialment desfavorable.
- La modulació del microbioma intestinal és factible i segura en el context de la quimioradioteràpia, però és pacient depenent i pot venir condicionada per l'estadi tumoral i altres factors endògens i exògens.
- L'estudi dels metabòlits circulants pot servir com a biomarcador per identificar pacients candidats i/o que responen a estratègies de modulació del microbioma intestinal.

Introduction

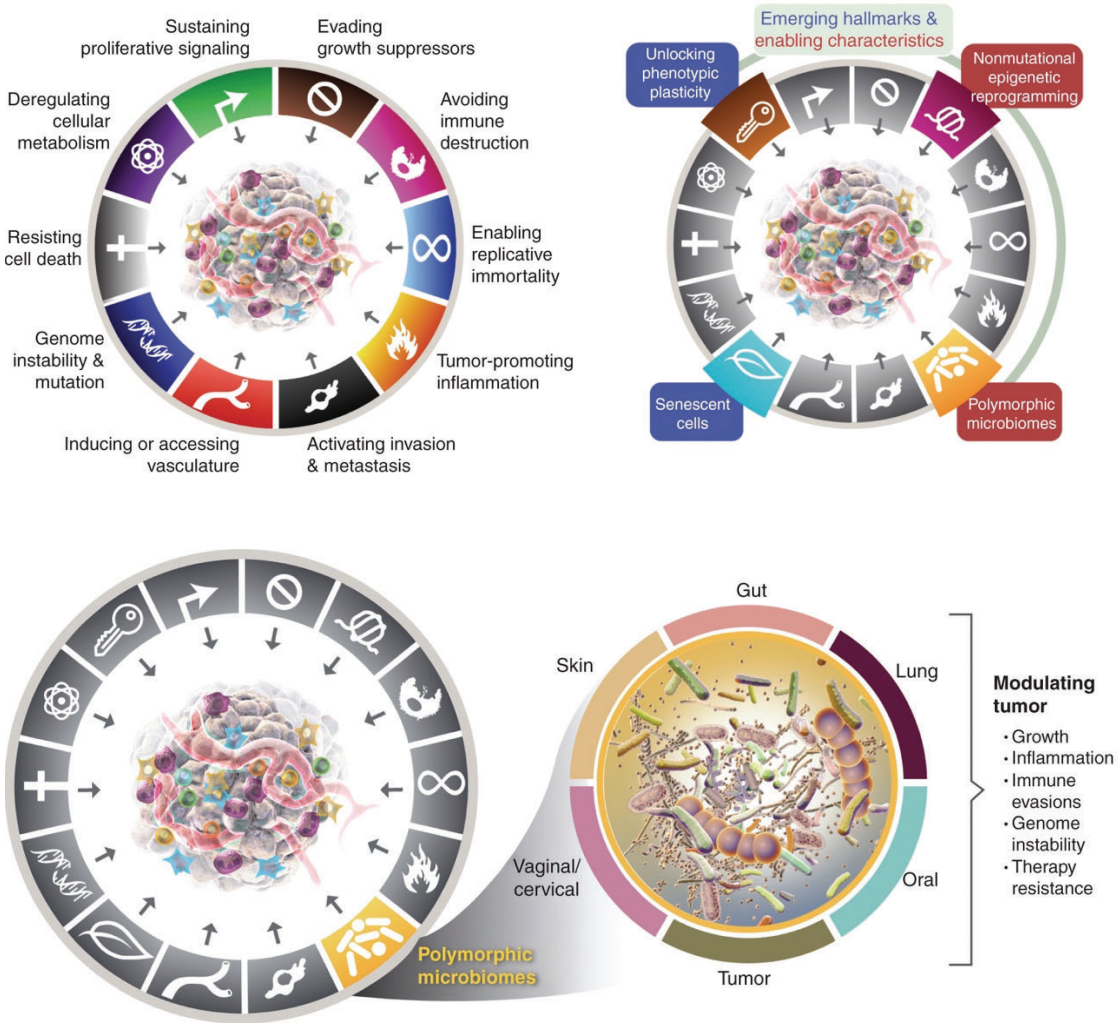
1.1. Microbiome as a new hallmark in cancer

The last ten years have been referred to as the "era of the microbiome," as Forbes noted in their 2019 article. The microbiome has long been known to be associated with human health, and in recent times, it has become increasingly important in several medical specialties, most notably oncology. The collective genomes and metabolic waste products of all the bacteria, viruses, fungi, protozoa, and archaea that live inside the human body are collectively referred to as the microbiome. These microbial communities are found in a variety of physiological compartments, including the gastrointestinal, urogenital, and cutaneous tracts(1). These communities retain essential functions like ATP generation, glycolysis, and the activation of translational machinery despite their diversity(1, 2). In a dynamic and symbiotic relationship with its human host, the microbiome is essential for controlling immunological responses and metabolic activities. Changes in a compartment's microbiome, whether from extrinsic or intrinsic sources such as genetics, infections, diet, or antibiotics, can cause chronic inflammation, upset local equilibrium, damage tissue integrity, and misalign systemic and local immune responses, all of which can result in disease(3-6). This state of imbalance, known as dysbiosis, has been linked to a number of illnesses, including cancer(7). There is a long-standing and well-established correlation between the microbiome and cancer, with infections responsible for 20% of cancer cases and certain pathogenic microbes playing a role in the etiopathogenesis of particular cancer types(8, 9).

In light of the accumulating evidence linking microbiome and cancer, the International Cancer Microbiome Consortium has proposed the microbiome as a crucial component of a tripartite model leading to carcinogenesis together with (epi)genetics and environmental variables(10). This idea is backed up by the Ecological Koch's postulates(11). The microbiome is becoming more widely acknowledged as a potential biomarker for cancer diagnosis, risk assessment, and prognosis, in addition to its role in cancer development. For instance, some tumor types have associated specific microbial signatures that can be detected in circulating cell-free DNA (12). Recently, the human microbiomes have been

incorporated as a new dimension in the updated Hallmarks of Cancer (**Figure 1**)(13). Furthermore, several associations between tumor-related microorganisms and patient response and outcome to anticancer therapies have been found(14, 15). Several studies have demonstrated how the gut microbiome influences host immune responses against cancer and the efficacy of immune checkpoint inhibitors (ICI) across different tumor types(16, 17). This has sparked research into the use of microbiome manipulation as a therapeutic approach to improve anti-cancer treatments through dietary changes, probiotic/antibiotic interventions, and fecal microbial transplantation (FMT)(18).

Figure 1. Microbiome as a new dimension in the hallmarks of cancer



Hanahan D, Cancer Discov. 2022;12(1):31-46

1.2. Impact of gut microbiome on immune response and antitumor immunity

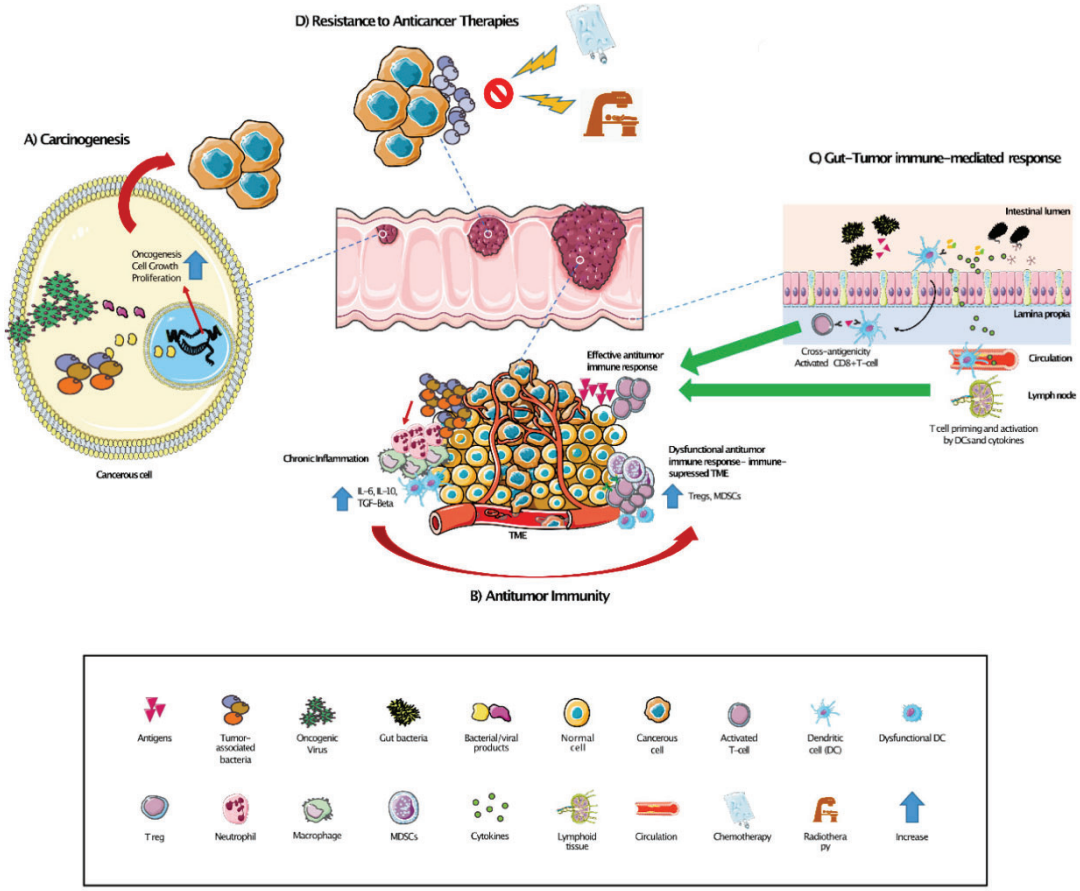
The intricate interaction between the gut microbiome and the immune system is critical for maintaining intestinal homeostasis, enabling a balance between tolerance to commensal microbes and the activation of immune defenses against pathogens. This dynamic interplay significantly influences the development and function of both innate and adaptive immune responses, guiding the maturation of myeloid and lymphoid cells(19-22). Gut microbiota modulate immune activities to elicit either anti-inflammatory or pro-inflammatory effects, depending on the specific immune cell types involved(23). For instance, certain bacterial species and their metabolic outputs can induce anti-inflammatory responses by promoting the differentiation of T regulatory cells, while others can trigger inflammation through the stimulation of dendritic cells, T helper cells, and CD8+ cells(24-27). This complex microbiome-immune system relationship is orchestrated by several mechanisms, including the recognition of microbial-associated molecular patterns (MAMPs) by toll-like receptors, which significantly affect the functionality and development of innate immune cells(28, 29). Bacterial products – metabolites - are pivotal in modulating the differentiation and polarization of these cells(24). Thus, the gut microbiome emerges as a fundamental contributor to the development of the immune system and its response modulation, influencing the etiology of various conditions including cancer, autoimmune diseases, and obesity.

The gut microbiome's impact extends to modifying immune-mediated anti-tumor responses, suggesting that microbial composition and diversity within the gut are crucial for the efficacy of immunotherapies across different cancer types, particularly ICI(16, 17, 30). These data evidences the potential of harnessing the gut microbiome to enhance tumor response to therapies, underlining the importance of further research into the detailed mechanisms linking gut microbiome profiles to tumor-immune system interactions. This advanced understanding could lead to innovative therapeutic strategies that leverage the microbiome for improved cancer treatment outcomes.

Beyond the gut, compartmental and/or tumor-associated microbiome composition might also play a role in local immune response and response to

anticancer agents, such as chemotherapy agents or immunotherapies (14, 31-33), although no data is available for the latter agents. Similarly to gut microbiome interplay, unraveling the exact link between tumor-associated microbiome and antitumor immune-responses will be crucial in order to tailor microbiome manipulation to boost antitumor responses in cancer patients (**Figure 2**).

Figure 2. Impact of gut and tumor-associated microbiome in cancer and antitumor response



(A)Carcinogenesis: intratumor bacteria and/or viruses and their by-products can activate oncogenic pathways and promote cell growth and proliferation. (B) Antitumor immunity: chronic inflammation caused by the local microbiome could lead to an immunosuppressive tumor microenvironment through altered antigen presentation and Tregs and myeloid-derived immunosuppressive cell simulation, ultimately impairing anti-tumor immune-responses. (C) Gut–tumor immune-mediated response: gut bacteria and their by-products can enhance CD8+ T cell-mediated antitumor responses via (1) cross-reactivity of shared bacteria and tumor antigens recognized by T cells in the gut; (2) activation of dendritic cells, which will lead to T cell priming and expansion; (3) local pro-inflammatory cytokines or other bacterial products entering systemic circulation along with activated T cells. (D) Resistance to anticancer therapies: intratumoral

bacteria can alter the efficacy of certain chemotherapies by altering the metabolism or through generating resistance to radiotherapy through hypoxic mechanisms.

Oliva M. Mulet-Margalef N., Ochoa de Olza M., Napoli S., Mas S., Laquente B. et al, Int J Mol Sci. 2021 Feb 1;22(3):1446

1.3. Gut microbiome predicts benefit to immune-checkpoint inhibitors

Extensive research has elucidated the pivotal role of the gut microbiome in modulating the efficacy of ICIs across a spectrum of malignancies. **Table 1** summarize most relevant and recent studies. These investigations have identified specific gut microbial configurations, termed "signatures," that correlate with a heightened immune infiltration within tumor microenvironments, particularly observed in patients with metastatic malignant melanoma undergoing therapy with anti-PD-(L)1 agents(16, 30). A notable correlation exists between increased alpha diversity of the gut microbiome—defined by both the species richness and evenness in the distribution of microbial taxa in fecal samples from patients—and improved therapeutic response and survival to anti-PD-(L)1 antibodies in the abovementioned studies. This enhanced response has been associated with a higher relative abundance of specific bacteria including *Clostridiales* order, the families of *Ruminococcaceae, Lachnospiraceae* and *Bifidobacteriaceae* and *Faecalibacterium prauznitzii*, *Ruminococcus spp*, *Mediterraneibacter spp*., and *Blautia spp*). In contrast, the gut microbiome of individuals who do not exhibit a response to ICI is characterized by diminished alpha diversity and a preponderance of the *Bacteroidales* order(34). Comprehensive analyses incorporating both gut microbiome composition and immunological evaluation of the tumor microenvironment have revealed enhanced expression of cytotoxic T lymphocyte markers and mechanisms involved in antigen processing and presentation in subjects possessing a beneficial gut microbiome profile(35). Routy et al. reported that baseline fecal samples from patients with non-small cell lung cancer and renal cell carcinoma, who exhibited a positive response to anti-PD-1 antibody therapy, were also enriched with *Akkermansia muciniphila* and various Firmicutes, both classified and unclassified(17). The same group confirmed in a subsequent larger cohort of patients with non-small cell lung cancer that *Akkermansia* was an independent predictive factor associated with increased response rates and overall survival (OS), regardless of PD-L1 expression, use of antibiotics, and performance status(36).

In conjunction with human cohort investigations, preclinical models demonstrate the potential for immune modulation via the transfer of exogenous microbiota into mice. The process of FMT from individuals who have shown a positive response to anti-PD1 antibody therapy into germ-free mice has been shown to reinstate antitumor immunity(17). This contrasts with the results from transplants using fecal material from non-responders, where no such immunological restoration was observed. The administration of *Akkermansia muciniphila* into mice receiving FMT from non-responders notably succeeded in re-establishing immunity to immune checkpoint inhibitor (ICI) therapy, indicating specific microbial species as critical drivers of immune response.

However, the search for definitive gut microbiome profiles predictive of ICI response remains ongoing, without universally recognized microbial "signatures." The relationship between specific bacterial taxa and therapeutic response is highly context-dependent, varying according to the patient cohort, experimental setup, and the particular bacterial species or strain involved. For instance, the administration of *Bacteroides fragilis* has been shown to restore responsiveness to anti-CTLA-4 therapy in germ-free and antibiotic-treated mice in some studies, while others have noted that patients with metastatic melanoma exhibiting a gut microbiome enriched in Bacteroides at baseline experienced shorter progression-free survival (PFS) and OS when compared to those with a Firmicutes-rich microbiota(34, 37). In a recent meta-analysis incorporating 5 melanoma cohorts including 147 stool samples treated with ICI, it was confirmed that baseline gut microbiome composition was associated with improved overall reponse rates (ORR) and PFS(38). However, the association was cohort dependent: the authors used artificial intelligence/machine learning analysis and found that the reproducibility of microbiome-based signatures across cohorts was limited. While a panel of species, including *Bifidobacterium pseudocatenulatum, Roseburia spp.* and *Akkermansia muciniphila*, was consistently associated with responders, but no single taxa could serve as biomarker in each of the separate cohorts.
Despite the amount of data generated, numerous questions remain. There is a pressing need for the standardization of experimental methodologies and data interpretation, which currently show significant variability across studies. The

transparent sharing of metadata and methodologies is essential for a comprehensive evaluation of the collective body of research and to contextualize findings accurately. The International Cancer Microbiome Consortium has recently highlighted the importance of this issue in a consensus statement, underscoring the necessity for collaborative efforts to elucidate the complex interplay between the gut microbiome and cancer immunotherapy outcomes(10)

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

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.

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1.4. How to explain the correlation between gut microbiome composition and ICI efficacy?

The elucidation of the molecular and cellular mechanisms underlying the observed correlation between the composition of the gut microbiome and the enhanced efficacy of ICIs remains an ongoing challenge in the field of cancer immunotherapy. Fessler et al. provided a comprehensive review on this subject, emphasizing the importance of identifying the intermediaries that convey signals from the gut microbiome to mediate tumor immune responses(39). These intermediaries, or "messengers," may originate from the microbiome itself—such as specific bacterial strains, MAMPs and/or pathogen-associated molecular patterns (PAMPs), and bacterial metabolites—or may be host-derived, including immune cells and cytokines. For instance, live bacteria or their associated molecular patterns can serve dual roles: as antigens that elicit T-cell mediated antitumor responses through cross-reactivity with tumor antigens or as adjuvants that enhance T-cell priming by activating antigen-presenting cells upon systemic translocation(37).Certain bacterial metabolites, like those produced by *Akkermansia muciniphila*, can influence cytokine production and T-cell differentiation, thus modulating the immune response against tumors and affecting the success of ICI therapy in preclinical models(40, 41).

Furthermore, host immune cells, particularly gut dendritic cells, play a critical role in mediating antitumor immunity(42). These cells are pivotal for maintaining immune tolerance towards commensal bacteria as well as for the priming, differentiation, and activation of T cells in response to specific bacterial strains or local mucosal inflammation(43). Tanoue et al. demonstrated that a consortium of 11 bacterial strains, typically present in low abundance in the human gut, can activate dendritic cells to induce interferon-γ-producing CD8 T cells within the gut, thereby enhancing the efficacy of anti-PD-1 antibodies in mouse models(27). These findings suggest not only a localized but also a systemic effect, with the phenotype of CD8 T cells varying across different organs, indicating a complex interplay between gut microbiome-derived signals and systemic antitumor immunity. Recently, the group of Griffin et al, found that a particular type of bacteria (enterococci) increase anti–PD-L1 agents' response in mice through secretion of an enzyme capable to break the bacterial cell wall and release

immunogenic peptides which in turn activate the innate immune response through the NOD2 pathway. These products can be detected in blood and potentially serve as circulating predictors of response regardless of microbiome composition(44).

1.5. Plasma and stool metabolomics as surrogate biomarkers of gut microbiome

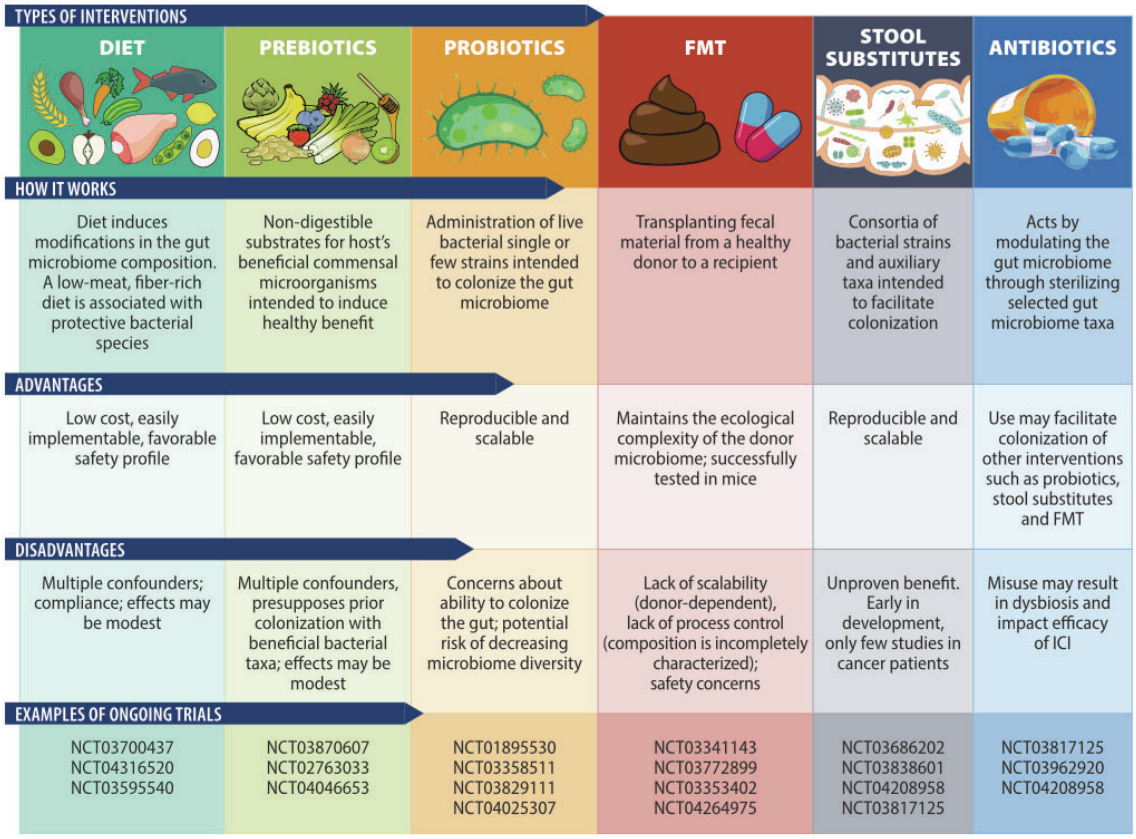
The gut microbiome's role in metabolizing a diverse array of substances within the gut lumen, encompassing xenobiotic food elements such as fiber, polyphenols, and amino acids, alongside smaller molecules like vitamins or pharmaceuticals and both host- and diet-derived lipids, including phospholipids and primary bile acids, is well-documented and can be detected in stool samples(45). A subset of these metabolites, synthesized exclusively by specific taxonomic groups within the gut microbiome, traverse into the systemic circulation and are identifiable in plasma samples. These metabolites can serve as indirect biomarkers of the gut microbiome's composition due to their taxonomically restricted origins. For instance, short-chain fatty acids (SCFAs), metabolites produced by gut bacteria from dietary fibers, are implicated as key regulators in T cell homeostasis, influencing T cell differentiation into effector or regulatory cells(24, 46). This modulation of T cell dynamics is critical for the efficacy of ICI, emphasizing the potential of these metabolites as therapeutic targets to modulate immune responses(46). Recent cohort studies have explored the potential of a specific plasma microbial metabolites as predictors for gut microbiome composition and its alterations. A study by Nomura et al. and Botticelli et al. has demonstrated that responders to immunotherapy had significantly higher fecal and plasma concentrations of SCFAs, highlighting the predictive value of these metabolites in assessing treatment response(47). Recently, stool microbiome composition and stool/plasma metabolome analysis MIND-DC phase III clinical trial evaluating adjuvant natural dendritic cell therapy in high-risk stage III melanoma showed that reduced cholic acid—a primary bile acid— and elevated levels of SCFA and acylcarnitines, correlated with decreased recurrence-free survival (RFS), particularly in the treatment group. Interestingly, the relative abundance of *F. prausnitzii* – which was associated with better RFS - anti-correlate with plasma bile acids and SCFA(48). Altogether, these findings

suggest host's microbiome composition and lipid metabolism, including carboxylic acids, bile acids, and acylcarnitines, may be pharmacodynamic markers of adjuvant immunotherapies, and indicates the potential applicability of metabolites as indicators of gut microbiome composition in individuals. However, it is crucial to prospective investigation of the predictive value of plasma microbial metabolites concerning the gut microbiome's state in patients receiving immunotherapies. Such studies could unveil novel biomarkers for monitoring therapeutic responses and microbiome dynamics, offering insights into the complex interactions between host metabolism, microbiome composition, and treatment outcomes.

1.6. Gut microbiome modulation strategies to improve antitumor immune responses

Recent advancements in understanding the gut microbiome's impact on cancer therapies have led to innovative strategies aimed at enhancing treatment efficacy and reducing adverse events. These strategies include dietary modifications, probiotics, prebiotics, selected antibiotics, and FMT (**Figure 3**). However, manipulating the microbiome is complex due to factors such as genetic predisposition, dietary habits, and concurrent medications, which can all influence microbiome composition and diversity. For instance, the use of Antibiotics, known to cause gut dysbiosis, have been shown to negatively affect both overall survival and progression-free survival in cancer patients, as well as impair responses and/or enhance toxicity to ICI (49-51). A brief summary of the current microbiome manipulating strategies and their current limitations is provided below.

Figure 3. Type of microbiome interventions to boost immunotherapy response



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Dietary changes, Prebiotics, Probiotics and Antibiotics

Dietary patterns influence the composition of the gut microbiota, and consequently have an impact on nutrient assimilation and the mediation of dietary advantages in humans(52, 53). The Mediterranean diet, characterized by its high fiber content and minimal red meat consumption, has been correlated with enhanced microbial diversity in contrast to the Western diet, which is high in animal fats and proteins and associated with reduced microbial diversity(54). Specifically, diets rich in protein have been linked to an increase in *Bacteroides* and *Clostridia* populations, with a concurrent decrease in *Bifidobacterium*, in comparison to plant-based diets(55). Dietary shifts can rapidly affect the gut microbiome's composition, with changes observable within 24 hours of diet alteration and a return to baseline within approximately 48 hours post-diet discontinuation(55).

Although these dietary modifications have demonstrated effects on immune responses in murine models(56), their potential to specifically amplify responses to anticancer therapies in cancer patients remains under exploration. Several ongoing clinical trials are examining the impact of dietary modifications and fiber supplementation as adjuncts to ICI therapy (**Figure 3**). However, the exact implications of adding or omitting specific nutrient classes (such as carbohydrates, proteins, or lipids) from the diet on microbiome composition and immune modulation are unclear and represent a significant area of research. This exploration is crucial for understanding the nuanced interactions between diet, the gut microbiome, and the immune system.

Changing the diet to influence the gut microbiome is cost-effective, easy, and generally safe. However, it's uncertain if diet alone can effectively prevent cancer or alter response to anticancer therapies. Tracking dietary changes is challenging due to various factors, and long-term adherence is also a concern. Recent efforts have been made to improve this approach, such as evaluating post-meal blood sugar levels (PPGR). PPGR is linked to several medical conditions, including cancer, and varies among people due to diet, physical activity, and gut microbiome differences(57, 58). A machine learning algorithm was developed to predict PPGR based on blood samples, diet, physical activity, and gut microbiome data(59). This approach showed promising results in predicting and improving post-meal blood sugar levels and modifying the gut microbiota composition. Incorporating methods like PPGR analysis to monitor dietary interventions is recommended for future research.

Prebiotics are described as substrates that host microorganisms preferentially use to provide a health advantage; these substrates are primarily nondigestible carbohydrates like fiber and resistant starch(55). Due to their resistance to digestion in the upper gastrointestinal system, these substances can pass through to the colon, where the gut microbiota ferments them. Beneficial bacterial genera including *Faecalibacterium* and *Bifidobacterium*, which, as mentioned before, have been connected to improved responses to ICIs in melanoma patients, are preferentially stimulated to flourish during this fermentation process(35). Prebiotics are safe and readily available as dietary supplements, providing an affordable approach to gut microbiota regulation. Preclinical studies

using syngeneic mouse models evaluating the co-administration of probiotics such as ginseng polysaccharides with antiPD-1 agents induced the production of metabolites and the suppression of regulatory T cells and induction of T-effector cells, leading to higher responses rates when compared to antiPD-1 agents alone(60).The potential benefit of prebiotic use in the clinical setting is yet to be elucidated.

Conversely, probiotics consist of live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. They are incorporated into various products, including foods, supplements, and pharmaceuticals. Probiotic research in colorectal cancer has highlighted that certain strains, like *Lactobacillus acidophilus* and *B. lactis*, can increase the abundance of butyrate-producing bacteria (particularly *Faecalibacterium* and other *Clostridiales*) within both the tumor and adjacent non-tumor colonic mucosa. Studies have also observed probiotics' ability to modulate mucosal immunity in colorectal cancer patients, evidencing changes in cytokine profiles indicative of an immunomodulatory effect(61). Multiple studies are currently evaluating the role of probiotics as an adjuvant for ICI treated patients (NCT03829111, NCT04025307). A metanalysis evaluating the impact of probiotic use on the survival of cancer patients treated with ICIs revealed increased responses and overall survival associated with probiotic use (62). However, the administration of probiotics must be approached with caution: 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(46). This study also assessed baseline dietary habits, and found that patients with a high fiber diet were more likely to respond to ICI.

Several studies have shown an association between the use of antibiotics prior to ICI treatments and poor responses and survival(63, 64). However, using antibiotics to change the gut microbiome before treatment with FMT, probiotics, or microbial combinations is a promising approach to optimize clinical outcomes. Preclinical and clinical studies indicate treatment with antibiotics may help restoring the microbiome following FMT or specific bacterial strains(65). In

regards to cancer patients, the MCGRAW randomized trial evaluated the impact of vancomycin pretreatment in patients with unresectable or metastatic melanoma naive to anti-PD1 therapy with the goal of priming the gut microbiome for the engraftment of SER-401, an oral microbial consortia(66). Preliminary results revealed poor outcome in the experimental arm, with overall lower engraftment than expected, which could be potentially attributed to the antibiotic regimen. As such, identifying the best timing as well as the appropriate antibiotic treatment is essential to maximize the therapeutic advantages of altering the microbiome.

Fecal microbiota transplantation (FMT) and Oral microbial consortia

FMT involves transferring complex communities of microbes, metabolites, and other fecal components from a healthy donor to a recipient, although the specifics are not fully understood. FMT has proven effective in treating primary and recurrent *Clostridioides difficile* infection, irritable bowel disease, and steroid-resistant colitis induced by ICIs(67-70). In preclinical studies, mice transplanted with fecal samples from ICI-responsive patients showed inhibited tumor growth(71). Currently, FMT is being investigated as an adjunct to ICI therapies in various cancer types through several clinical trials (NCT03353402, NCT04264975, NCT03341143, NCT03772899, NCT04130763, NCT04116775, NCT04056026). Additionally, FMT is being explored to mitigate and prevent treatment-related side effects (NCT04163289, NCT03772899, NCT03819296). Two phase 1 studies have shown encouraging results. Baruch et al. performed a phase I clinical trial to assess the safety, feasibility, and immune cell impact of FMT plus anti-P-D1 in PD-1 in refractory metastatic melanoma patients. Interestingly, this combination appeared safe and induced radiological tumor responses and tumor immune infiltration by CD8+T cells(72). The group of Routy et al. evaluated the same combination but as first-line therapy in ICI-naïve melanoma patients. The treatment was proven to be safe and tolerable and responses were promising compared to historical results with ICI alone, but most importantly, longitudinal stool samples revealed enrichment of immunogenic and a loss of deleterious bacteria following FMT among the responders (73).

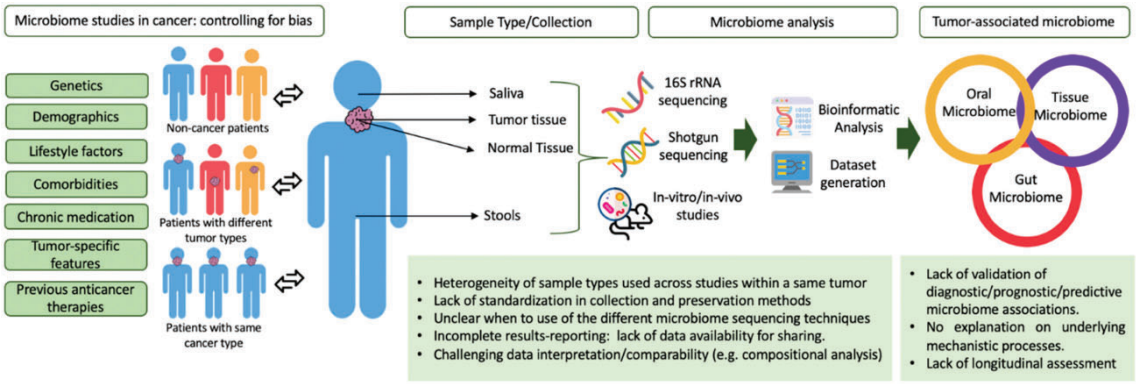
However, despite encouraging results, FMT has limitations, including: lack of control over the process due to the unknown and variable bacterial composition from stool donations; difficulty in producing consistent therapeutic stool on a large scale; and safety concerns related to the potential transmission of known or unknown pathogens and host-associated traits. FMT has been associated with the death of two patients receiving treatment for *Clostridium difficile* colitis due to the development of antibiotic-resistant microbes. As a result, the US Food and Drug Administration issued a cautious warning to FMT researchers (74).

As an alternative to FMT, oral bacterial consortia – a mixture of pure live cultures of bacteria, often isolated from a stool sample of a healthy donor – have been developed and are under evaluation in clinical trials (**Figure 3**). Some of these consortia such as Microbial Ecosystem Therapeutics (MET) have been tested to treat *Clostridioides difficile* infection with encouraging results (75, 76). In cancer patients, tested consortia often include multiple species that have been previously correlated with increased efficacy of ICI in cancer patients or based on their ability to elicit systemic CD8+ T-cell responses. A few examples include SER-401 (NCT03817125), Microbial Ecosystem Therapeutics (MET)-4 (NCT03686202) or VE800 (NCT04208958). Some the early studies evaluating the abovementioned agents have already results available and are discussed in the different sections of this thesis.

1.7. Challenges in conducting microbiome studies in cancer patients

The International Agency for Cancer Research has highlighted significant variability among microbiome studies regarding their description methods, techniques employed, taxonomic depth, and information on confounding factors(77). There is a pressing need for standardizing methodologies and reporting results, as well as controlling biases in studies related to the microbiome. **Figure 4** outlines the current challenges faced by microbiome studies in cancer research.

Figure 4. Challenges of microbiome studies in cancer



Oliva M. Mulet-Margalef N., Ochoa de Olza M., Napoli S., Mas S., Laquente B. et al, Int J Mol Sci. 2021 Feb 1;22(3):1446

Collection, Preservation and Sequencing approaches

Microbiome analyses can be conducted in various biological sample types, such as tumor tissue, body fluids, or stools, each with distinct collection and preservation methodologies, leading to variable results. For example, gut bacterial communities exhibit different diversities when analyzed using stool samples compared to intestinal mucosal tissue, despite homogeneous distribution along the colon mucosa(78). The selection of sample type and preservation methods is crucial when investigating the tumor-associated microbiome. While microbiome composition differences are anticipated between distinct body compartments, the representativeness of tumor tissue versus samples from the cancer-associated compartment remains unclear.

In colorectal cancer studies, stool samples may not fully capture colorectal-associated microbial communities(79). Conversely, in head and neck cancer and urothelial cancer studies, similar microbiome compositions and diversities were observed across saliva, tumor tissue, tumor swabs, urine, and tumor tissue (80, 81). Nevertheless, further studies are warranted to validate these findings.

Optimal sample handling and preservation are essential to prevent bacterial proliferation and contamination. Various studies have examined the stability and variability of microbiome diversity and composition under different timeframes and preservation temperatures(81). The International Human Microbiome Standards consortium has provided guidelines and standard operating

procedures for sample collection, considering processing times and freezing capabilities(82). For transcriptomic analyses, RNAlater can be employed, albeit potentially affecting DNA yield(83).

Type of sample collected and processing may impact on the sequencing approach and ultimately on the results obtained.

Sequencing techniques and bioinformatic analysis

There are different sequencing and bioinformatic analysis approaches to study the human microbiome, each with its advantages and disadvantages (**Table 2**).

Table 2. Methodology for microbiome analysis: problems and solutions

Type	Technique	Problem	Solution/Alternative
Sequencing technique	16S rRNA-seq	Low taxonomic resolution Limited functional analysis	Full-length 16S sequencing, shotgun sequencing
	Whole shotgun sequencing	More expensive Human DNA also gets sequenced	Sequencing at low coverage Adequate source material, enrichment of microbial material before sequencing
	Long read sequencing	Sequencing errors are difficult to detect	Combining long read sequencing with short read shotgun
16S bioinformatics	OTU-based methods	Loss of information in clustering	ASV-based methods
	ASV-based methods	Reliance on the algorithm to detect sequencing errors	
Shotgun bioinformatics	Taxonomic profiling	Reliance on incomplete databases	New assemblies will provide more complete databases
	Functional profiling	Reliance on incomplete databases, proteins of unknown function	Further characterization of microbial proteins is still needed
	De novo assembly	Incomplete assemblies, chimeric genomes, strain heterogeneity	Strict quality control Long-read sequencing will provide better assemblies
Biostatistics	Traditional statistics	Datasets are compositional	Compositional methods, estimation of total microbial presence to avoid compositionality
	Compositional analysis	Presence of zeroes Difficult to interpret	Zero-replacement
Spatial in situ resolution	RNA in situ hybridization	Low-throughput (only 2-3 bacterium can be detected)	Use it when information about spatial resolution is needed

Oliva M. Mulet-Margalef N., Ochoa de Olza M., Napoli S., Mas S., Laquente B. et al, Int J Mol Sci. 2021 Feb 1;22(3):1446

The primary sequencing methods employed to characterize taxonomic relative abundance (RA) are high-throughput 16S ribosomal RNA gene amplicon sequencing (16S rRNAseq) and whole shotgun metagenomics(84). 16S rRNAseq involves amplifying specific variable regions (V3-V4) of the bacterial 16S rRNA gene via PCR, offering cost-effective microbiome characterization with

genus-level resolution(85). Alternatively, long-read sequencing of the complete 16S rRNA gene can provide higher taxonomic resolution(86). In contrast, whole shotgun metagenomics sequences the entire DNA content in samples, enabling identification of species and genes across all microorganisms, not limited to bacteria, given sufficient sequencing depth. The PCR amplification step in 16S sequencing ensures targeted microbial DNA analysis will be sequenced. This is not the case for shotgun sequencing, where the DNA samples need to be enriched for microbial DNA beforehand.

Bioinformatics analysis of 16S rRNA gene sequencing traditionally involves clustering similar reads to a predefined level of similarity, typically 97%, to form operational taxonomic units (OTUs). While this clustering mitigates sequencing errors, it also leads to information loss. Novel methodologies aim to preserve all amplicon sequence variants (ASVs) by algorithmically differentiating sequencing errors from biological variations [87, 88]. QIIME2 serves as a comprehensive bioinformatics toolkit, offering frameworks for integrating various steps of 16S rRNA analysis [89]. In shotgun metagenomics, multiple analytical approaches are available. Read-based classification algorithms endeavor to taxonomically assign each sequencing read, enabling qualitative and/or quantitative microbial profile analysis. Various software implementations support this task [90]. Additionally, reads can be functionally classified into gene families, offering insights into toxicity gene identification, pathway reconstruction, etc. Both approaches are constrained by database contents. De novo assembly of shotgun metagenomics reads facilitates genome reconstruction without database dependency, allowing for the discovery of novel genomes. In microbiome modulation studies, such as oral bacterial consortia applications, metagenomic analysis aids in distinguishing between exogenous and endogenous taxa, providing direct evidence of engraftment.

A primary limitation of 16S rRNA sequencing and metagenomic analyses is the lack of spatial information regarding community distribution within samples, precluding comprehensive understanding of bacterial interactions within the microenvironment or among themselves. Fluorescence in situ hybridization (FISH) and RNAscope offer alternative methods for direct visualization of RNA in

formalin-fixed, paraffin-embedded (FFPE) tissues, enabling sensitive and specific spatial analysis of all RNA molecules present in a sample simultaneously [91].

Statistics for Microbiome Analysis

The computation of diversity metrics is frequently employed in taxonomic profile analyses. Diversity indices, such as the Shannon and Simpson indices, assess within-sample diversity, also known as α-diversity, while β-diversity metrics, including Bray-Curtis and UniFrac, evaluate between-sample diversity. In addition to diversity assessments, standard statistical methods may be utilized to identify significant differences between groups. However, it is crucial to recognize that microbiome datasets derived from sequencing are compositional in nature. They offer relative rather than absolute descriptions of the microbiome within each sample, necessitating specialized statistical approaches [87]. This complexity complicates result interpretation and increases the risk of identifying spurious associations without appropriate methodology. Alternatively, quantifying the total microbial load circumvents compositional issues and has been demonstrated to yield deeper insights [88].

Controlling for Bias

Observational studies investigating the tumor-associated microbiome can be categorized into two primary types: (1) case-only studies, which evaluate the tissue microbiome composition concerning cancer prognostic events such as treatment response or resistance, tumor recurrence, and mortality; and (2) case-control studies, which compare tissue microbiome profiles between cancer patients and cancer-free individuals. Case-only studies focus on longitudinally tracking prognostic events, necessitating meticulous consideration of potential influencing factors on microbiome composition, including tumor clinical features, medication usage, demographic variables, and lifestyle choices. Accurate assessment of these factors, particularly dietary intake, is imperative for study design. Ideally, microbiome analysis should be performed on tissue samples collected prior to any therapeutic intervention.

Conversely, case-control studies aim to investigate tissue microbiome composition as a potential cancer risk determinant by contrasting cancer patients with healthy controls. These studies encounter challenges such as potential biases in study execution and the difficulty in procuring normal tissues from cancer-free individuals for comparative analysis. While normal mucosal tissue from cancer-free individuals can be obtained via colonoscopy, offering insights into microbiome composition disparities, caution is required when interpreting these differences due to potential variations in age, health status, and other variables between tissue-bank or donor samples and tumor tissues. Although saliva or stool samples have been utilized as surrogates for tissue microbiomes in assessing cancer risk, the causal relationship between microbiome composition and cancer development remains to be conclusively established. Prospective cohort studies that collect microbiome samples prior to disease onset are essential to validate observed associations from retrospective case-control studies. Replication of association signals across additional studies and further mechanistic investigations through in vivo studies are pivotal for advancing understanding.

Moving 1 or 100 steps forward: clinical trials in cancer patients

As the field of microbiome research in oncology evolves, it presents unique opportunities for leveraging this knowledge towards therapeutic interventions in cancer treatment. The design of clinical trials exploring the microbiome as an interventional strategy necessitates adopting both proof-of-mechanism and proof-of-concept frameworks. Proof-of-mechanism studies are essential for elucidating the direct effects of microbiome manipulation on the host immune response and tumor microenvironment. These investigations should meticulously account for variables such as the influence of anticancer treatments, antibiotic use, and dietary factors, and involve the collection of tumor tissue samples for detailed immune contexture analysis. The preoperative period, offering direct access to tumor tissues, is particularly conducive to such studies, though it presents challenges regarding the short duration of microbial interventions and the potential oversight of delayed effects.

In contrast, proof-of-concept studies aim to evaluate clinically significant outcomes like tumor response, PFS, or OS, contingent upon prior confirmation of the safety and feasibility of microbiome modulation. These studies are particularly pertinent to patient cohorts encountering resistance to immunotherapy, where randomized controlled trials can shed light on the efficacy of microbiome-based interventions. Despite the inherent challenge in controlling for confounders such as antibiotic use and dietary variations, meticulous data collection in these domains is imperative for the robust interpretation of trial outcomes.

The imminent proliferation of microbiome-related clinical trials in oncology demands rigorous standardization across various aspects, including sample collection, endpoint reporting, and the interpretation of microbiome alterations in the context of clinical responses. Establishing these standards is critical for enhancing the reliability and interpretability of research findings in this burgeoning field, ultimately aiming to integrate microbiome insights into the cancer treatment paradigm effectively.

1.8. Preliminary work of the PhD applicant on microbiome modulation in cancer patients: The MET-4-IO clinical trial

A randomized single-center investigator-initiated clinical trial was designed to evaluate the safety, tolerability and stool engraftment of MET-4 in patients with advanced solid tumors receiving ICIs (antiPD-(L)1 and antiCTLA-4 agents) (NCT03686202)(87). MET-4 is a modified version of the previously described microbial ecosystem therapeutics (MET) compound - an orally delivered defined mixture of pure live cultures of intestinal bacteria isolated from the stool of a healthy donor, purified and grown in conditions modeling those of the human distal gut - composed of 30 phylogenetically and functionally diverse bacterial species including taxa previously associated with ICI responsiveness (**Figure 5**). The study was conducted at Princess Margaret Cancer Centre (Toronto, Canada) and included a total of 40 patients in 3 separate cohorts: A. A safety cohort of patients with advanced solid tumors to be treated with ICI as per standard of care: all of them were planned to receive MET-4; B and C. an ICI-naïve (B) or pre-exposed (group C) patients, randomized to receive either standard-of-care ICI alone or in combination with MET-4 (**Figure 6**). The primary endpoints included

cumulative relative abundance of MET-4 taxa following 10 to 16 days of intervention (T1), changes in relative abundance of MET-4 taxa between MET-4 administration (T0) and T1 and treatment-related adverse events (AEs) assessed by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI- CTCAE) v5.0. For the ecological co-primary endpoint, patients were considered assessable if stool samples were obtained at T0 and T1 (a total of two stool samples in groups A and C and three stool samples in group B). Secondary endpoints included cumulative relative abundances of MET-4 taxa at T2-T4, changes in relative abundance of MET-4 taxa between baseline (T0) and post-randomization timepoints and bacterial taxonomic diversity between T0 and T2-T4. Exploratory outcome measures included overall response rate measured as per RECIST v1.1. Methods are described in the full publication provided.

Figure 5. MET-4 compound by Nubyota

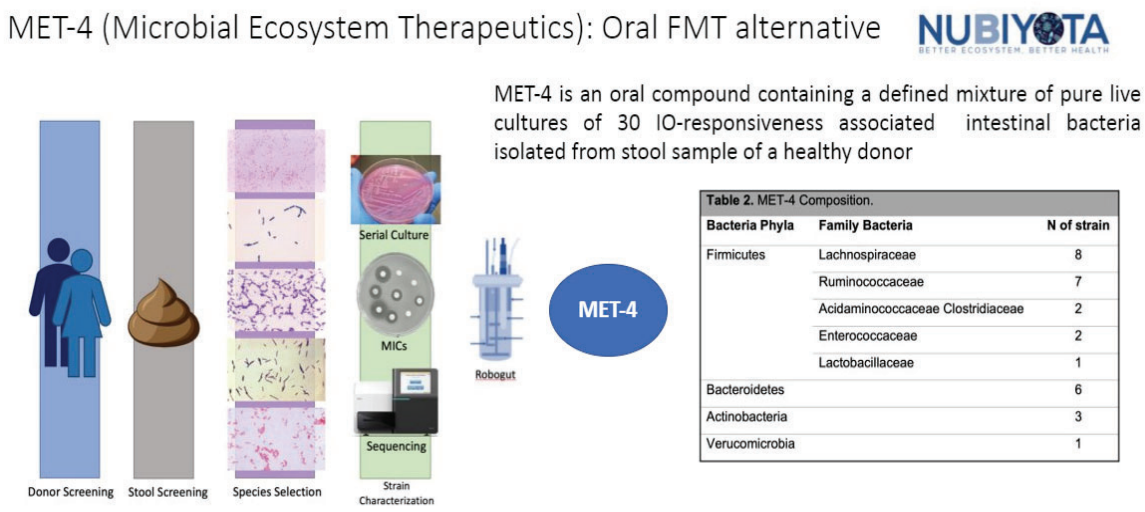
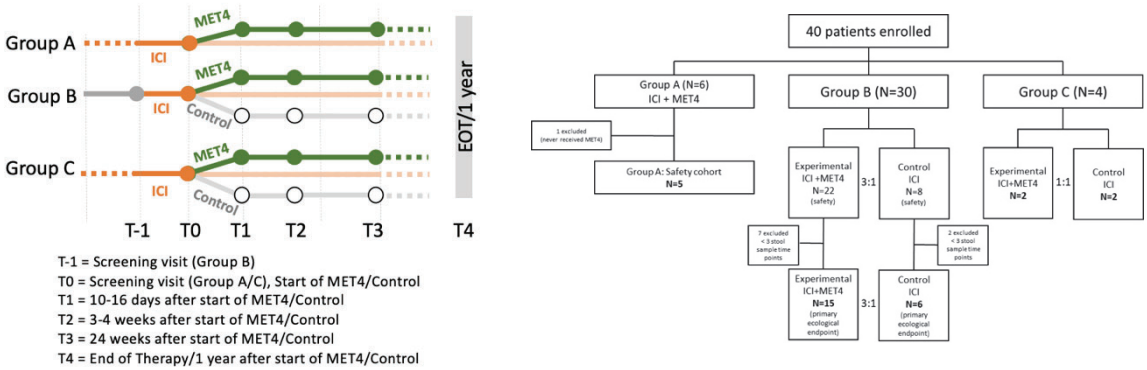


Table 2. MET-4 Composition.

Bacteria Phyla	Family Bacteria	N of strain
Firmicutes	Lachnospiraceae	8
	Ruminococcaceae	7
	Acidaminococcaceae Clostridiaceae	2
	Enterococcaceae	2
	Lactobacillaceae	1
Bacteroidetes		6
Actinobacteria		3
Verucomicrobia		1

Figure 6. Study design and consort diagram



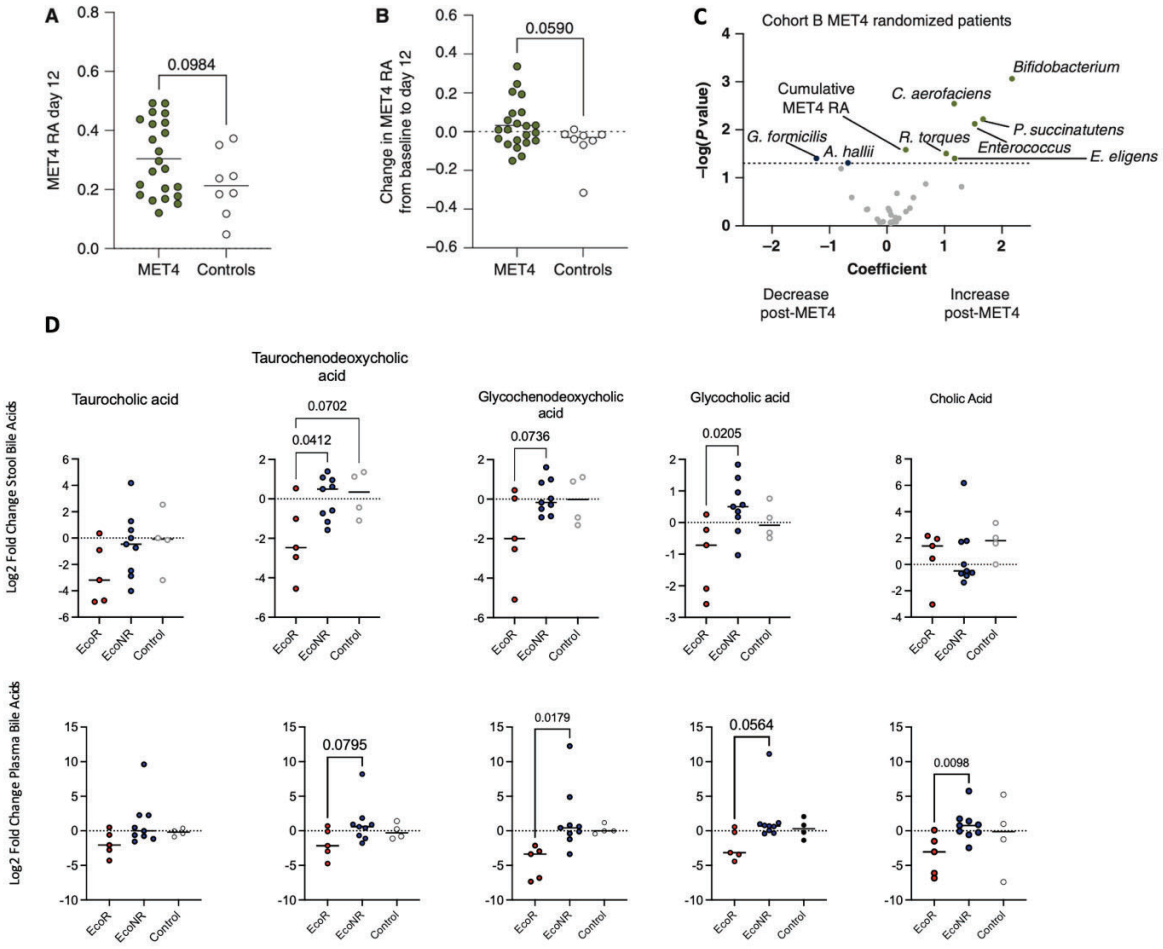
Spreafico A., Heirali A. A, Araujo D.V., Tan TJ, **Oliva M**, Schneeberger PHH et al. Ann Oncol 2023 Jun;34(6):520-530

Regarding the safety primary endpoint, there were no differences in between the MET-4 and control groups with respect to the number of immune-related AEs of any grade or grade $\geq$ 3 only and overall MET-4 was well-tolerated, with attributed AEs only occurring in 17% (5/29) of patients, were mainly gastrointestinal (including bloating, constipation, diarrhea, dyspepsia anorexia and weight loss), and only of mild/moderate severity (grade 1- 2).

As of ecological primary endpoint, there were no statistically significant differences although a trend towards increased MET-4 relative abundance was observed at T1 compared to controls (**Figure 7A-B**). MET-4 species relative abundance was evident after randomization but varied by patient and species. Increases in the relative abundance of several MET-4 taxa, including *Enterococcus* and *Bifidobacterium*, taxa previously associated with ICI responsiveness, were observed (**Figure 7C**). Exploratory metabolomic analysis revealed MET-4 engraftment was associated with decreases in plasma and stool primary bile acids (**Figure 7D**). Although exploratory, overall RECIST response rate was higher in MET-4 recipients vs controls in cohort B: 35% (6/17) versus 14% (1/7) respectively, although did not reach statistical significance (p value= 0.37). The association between ecological responsiveness and clinical response was not assessed in this early-phase trial, especially given the limited number of patients and the heterogeneity in the enrolled patient population (multiple tumor types and different ICI regimens).

Despite the additional inherent limitations of microbiome evaluation (patient intrinsec and extrinsec variability, 16SRNA sequencing not allowing to differentiate endogenous vs exogenous MET-4 taxa) and the impossibility to correlate ecological endpoints with changes in the tumor immunecontexture (ie. T-cell infiltration) due to the lack of paired tumor-biopsies, this trial is the first report of the use of a microbial consortium as an alternative to fecal microbial transplantation to modulate gut microbiome in advanced cancer patients receiving ICI and the results justify the further development of microbial consortia as a therapeutic co-intervention for ICI treatment in cancer.

Figure 7. Ecological endpoints



Stool samples were collected at 3-4 weeks post-ICI/pre-MET-4 (T0) and at four prespecified timepoints (day 12 post-MET-4/T1, week 3-4 post-MET-4/T2, week 24 post-MET-4/T3 and at the end of therapy or 1 year/T4) after randomization to receive MET-4 or standard-of-care ICI. 16S rRNA gene sequencing was used to determine: (**A**) cumulative RA of MET-4 taxa and (**B**) change in cumulative MET-4 RA. **C.** Volcano plots depicting differentially abundant MET-4 taxa post-randomization compared to samples collected before randomization: Increase (green) and decrease (blue) in MET-4 taxa post-MET-4 initiation. Grey dots include features that were not significantly different. MET-4 taxa and alpha diversity metrics were log transformed and analyzed using MaAsLin2. Fixed effects included MET-4 versus control randomization and pre- versus post-treatment; patient was set as a random effect to account for repeated measures. **D**. Plasma bile acid levels in MET-4-IO trial participants. B – Log2-Fold change between T2 and T0 in plasma bile acids for individuals with samples available at both timepoints, stratified by ecological response (EcoR, defined as >10-fold increase in relative abundance of >5 taxa between timepoints) or non-response (EcoNR) to MET-4, and controls are shown. All p-values <0.10 shown are post-tests of ANOVA comparing all groups.

Spreafico A., Heirali A. A, Araujo D.V., Tan TJ, **Oliva M**, Schneeberger PHH et al. Ann Oncol 2023 Jun;34(6):520-530

1.9. Human papillomavirus-related as an etiological and prognostic factor in oropharyngeal squamous cell carcinoma.

HNSCC represent the seventh most common malignancy worldwide, with approximately 890,000 new cases and 450,000 deaths annually as of 2020, according to Globocan 2020 data(88). The three main risk factors for developing HNSCC are alcoholism and tobacco use and oncogenic viral infection by the HPV(89). While head and neck cancers comprise a variety of malignancies affecting the oral cavity, oropharynx, hypopharynx, larynx, nasal cavity and paranasal sinuses as well as salivary glands, the etiopathological role of HPV has majorly been linked to OPSCC(90). In particular, the incidence of OPSCC attributable to HPV has been progressively increasing over the past 2 decades(91, 92). Globally, the percentage of OPSCCs that are HPV-related was reported in 2021 to be 33%; however, prevalence varies considerably depending on the geographical region, with estimates ranging from 0% in southern India to 45% in Italy and 70% in Northern Europe (93). The current trends in incidence and prevalence of HPV-related OPSCC over HPV-unrelated HNSCC reflect a change in lifestyle, such as the decrease in smoking and alcohol habits but also the patterns of sexual behavior, in particular the increase in number of lifetime oral sex partners, which has been identified as a critical risk factor for HPV-related OPSCC(94). The rise in HPV-attributable fraction has been more pronounced in high-income developed countries, particularly in Canada, USA and North Europe, likely due to differences in sexual practices as well as decreased tobacco use. While HPV+ OPSCC was previously more common in younger adults, recent trends show a shift towards older age groups, with a significant portion of cases now being diagnosed in individuals aged 65 years and above(95).

HPV-related OPSCC are a biologically distinctive disease characterized by increased radiosensitivity and improved overall survival when compared with HPV-unrelated OPSCC, which behaves similar to other tobacco/alcohol related HNSCC(96-98). This disparity in prognosis was not initially captured in American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) staging system and led the head and neck community to develop new staging criteria for HPV-related OPSCC(99). The 8th edition TNM (TNM8) provides a more accurate prognostic classification in order

to drive a better patient selection and tailored therapeutic approach. The molecular biology and genomic features of HPV-related OPSCC are shared with other HPV-associated malignancies but differ from HPV-unrelated OPSCC, with HPV oncogenes (E6 and E7) acting as key drivers of pathogenesis and most commonly molecular alterations involving DNA damage response proteins, FGF and JAK–STAT signaling proteins and PIK3CA and HLA-A/B genes(90, 100, 101). Table summarizes main differences between HPV-related and unrelated OPSCC.

In the curative setting, treatment de-intensification strategies are being pursued in clinical trials in order to reduce therapy-related acute and long-term toxicity and morbidity while achieving the best oncologic outcomes. De-escalation strategies include organ-preservation minimally-invasive surgery approaches, reduction of radiotherapy dose and sparing the use of cisplatin chemotherapy(102). Despite retrospective pooled-based analysis have suggested that cisplatin cumulative dose does not seem to affect the outcome in patients with HPV-related OPSCC treated with chemoradiotherapy, it's yet to be determined whether it can be avoided at all(103, 104). Most chemo-sparing strategies involve the substitution of cisplatin using other systemic agents such as targeted therapies (ie. Cetuximab) or immunotherapies (i.e checkpoint blockade with antiPD(L)-1 and antiCTLA-4 agents)(105). While the use of cetuximab as a radiosensitizer instead of cisplatin concurrent to radiotherapy has been shown to be detrimental in terms of locoregional control and survival in two large phase 3 studies, immunotherapy de-escalation studies are still on-going and results are awaiting(106, 107) (NCT03952585, NCT03410615).

Table 3. Comparison of key characteristics of HPV-related and unrelated OPSCC

Characteristics	HPV⁺ OPSCC	HPV⁻ OPSCC
Patient characteristics		
Average age at diagnosis (years)	59[a]	60 ($P<0.001$)[38]
Sex	86.9% male	76.8% male ($P<0.001$)[38]
Ethnicity	90% white	75.9% white ($P<0.001$)[38]
Role of smoking	Rising incidence of HPV⁺ OPSCC in smokers, as well as in nonsmokers[38]	
Role of alcohol	HPV⁻ OPSCC associated with greater alcohol consumption[7]	
Role of sexual history	High number of sexual partners a risk factor for HPV⁺ OPSCC[7]	
Tumour characteristics		
Incidence per 100,000	4.62	1.82 (REF.[38])
Anatomical location	More prevalent in oropharynx (94.2% HNSCC); specifically the base of tongue and tonsils[2]	Less prevalent in the oropharynx (72.8% HNSCC)[38]
Stage (AJCC 7th edn)	Early stage (T1–2); frequently with nodal metastasis at presentation[156]	All stages (T1–4)[38]
Histopathological appearance	Immature, basal-like/basaloid, non-keratinizing[156]	Frequently keratinizing SCC
Cancer-specific mortality	HPV⁺ OPSCC associated with a more favourable prognosis (aHR 0.40, $P<0.001$)[38]	
Biological characteristics		
Genetic alterations	More frequent alterations in genes encoding DNA damage response proteins, FGF and JAK–STAT signalling proteins, as well as immune-related genes such as *HLA-A/B*; *PIK3CA* mutations more commonly observed[95]	Aberration of *TP53* and cell-cycle pathways (such as *CDKN2A* loss); oxidative stress regulation more frequently mutated[95]
Other aberrations	p53 and Rb degradation by E6 and E7, respectively[243]	NR

[a]Incidence of human papillomavirus-positive oropharyngeal squamous cell carcinoma (HPV⁺ OPSCC) increasing in older men. AJCC, American Committee on Cancer; aHR, adjusted hazard ratio; HNSCC, head and neck squamous cell carcinoma; NR, not reported; OPSCC, oropharyngeal squamous cell carcinoma; SCC, squamous cell carcinoma.

Lechner M, Liu J, Masterson L, Fenton TR. Nat Rev Clin Oncol. 2022 May;19(5):306-327

1.10. Biomarkers for risk-stratification in HPV-related OPSCC

One of the key challenges in the era of de-escalation is to identify those patients who truly benefit for these strategies. Despite a favorable prognosis, up to 20% of patients with locally-advanced HPV-related OPSCC will eventually recur, mostly in the form of distant metastasis(108). While the implementation of TNM8 helped in stratifying patients with higher vs low risk of recurrence (stage III vs I and II, respectively), it is not accurate enough. Other factors beyond disease burden are at play. Smoking, for instance, is known to have a negative impact in overall survival in this patient population(96, 109). Retrospective analyses of heterogeneous cohorts of patients with LA-OPSCC treated with different treatment modalities, smoking pack-year has been correlated with worse OS and distant control(108, 110). Based on this, experts in the field have proposed a risk-stratification based not only on stage but also on smoking pack-year(98).

However, the role of smoking in the biology of the disease remains controversial, as some studies have failed to show a correlation with cancer-specific survival and disease-control outcomes(111). Smoking has a direct impact on overall health, and comorbidities are associated with decreased survival in HNSCC regardless of treatment intervention and stage(112). Among patients with HNSCC, smokers are at higher risk of developing secondary malignancies, especially younger patients who more frequently present with HPV-related OPSCC(113, 114). In addition, smoking is known to affect radiotherapy efficacy and toxicity, which may ultimately affect response and disease-free survival(115). The PhD applicant evaluated the role of cumulative cisplatin and smoking pack-year in a cohort of 482 patients with HPV-related OPSCC treated with chemoradiotherapy(116). The 5-year cancer-specific survival in the whole cohort and by staging subgroups was unaffected by smoking pack-year (using 10, 20, and 30 pack-year cut-off). When age, stage, and cisplatin dose were taken into account, no consistent findings were found in the multivariate analysis (**Table 3**). In contrast, regardless of stage, patients with smoking pack-year $\geq$30 versus <30 had a substantially worse 5-year OS (75% versus 88%, p value=0.017) and smoking pack-year (continuous by 10) showed a negative effect on OS in the multivariate analysis after adjusting for age, stage, and cisplatin dose (HR: 1.14 [95% CI: 1.02e1.27] p value=0.01). The implications of smoking in carcinogenesis and immunosuppression are also well-known, but the role that tobacco plays in the biology of HPV-related OPSCC has not yet been elucidated and the analysis from studies comparing the tumor genomic and immune landscapes in smokers versus non-smokers have been inconsistent thus far(117, 118).

Table 4. Impact of smoking in HPV-related OPSCC

Impact of cisplatin dose and smoking pack-year on OS and CSS in the entire cohort and stratified by stage.

CDDP-D (mg/m^2)		Entire cohort (N = 482)	Stage (TNM8)		
			I (N = 189)	II (N = 174)	III (N = 119)
OS	**5-year OS (95% CI)**				
	<200	82% (75–91)	88% (78–100)	86% (75–98)	65% (47–89)
	=200	88% (83–93)	94% (88–99)	83% (74–92)	89% (81–99)
	>200	86% (80–92)	95% (89–100)	84% (74–96)	74% (61–89)
	p value	*0.31*	*0.13*	*0.81*	*0.09*
	MVA HR (95% CI)				
	=200 versus < 200	0.66 (0.38, 1.16 *p=0.15*)	0.57 (0.18, 1.75 *p=0.32*)	1.20 (0.49, 2.97 *p=0.69*)	0.36 (0.13, 1.03 *p=0.05*)
	>200 versus < 200	0.74 (0.41, 1.35 *p=0.33*)	0.31 (0.07, 1.34 *p=0.12*)	0.92 (0.32, 2.64 *p=0.88*)	0.91 (0.38, 2.18 *p=0.83*)
	≥200 versus < 200	0.70 (0.42, 1.15 *p=0.16*)	0.46 (0.16, 1.31 *p = 0.15*)	1.10 (0.46, 2.58 *p=0.84*)	0.61 (0.27, 1.36 *p=0.22*)
CSS	**5-year CSS (95% CI)**				
	<200	89% (81–94)	98% (84–100)	88% (70–96)	**76% (49–89)**
	=200	91% (86–94)	95% (87–98)	84% (72–90)	98% (85–100)
	>200	88% (82–93)	95% (84–98)	84% (69–92)	84% (67–93)
	p value	*0.66*	*0.74*	*0.86*	***0.02***
	MVA HR (95% CI)				
	=200 versus < 200	0.79 (0.38, 1.63 *p=0.53*)	2.59 (0.25, 26.9 *p=0.42*)	1.39 (0.52, 3.77 *p=0.51*)	**0.08 (0.01, 0.67 *p = 0.02*)**
	>200 versus < 200	1.11 (0.52, 2.34 *p=0.79*)	2.89 (0.23, 35.6 *p=0.41*)	1.03 (0.42, 4.08 *p=0.65*)	0.76 (0.2, 2.23 *p=0.62*)
	≥200 versus < 200	0.92 (0.48, 1.77 *p=0.80*)	2.71 (0.27, 26.98 *p=0.39*)	1.36 (0.53, 3.51 *p=0.52*)	0.38 (0.13, 1.07 *p = 0.066*)
Smoking PYs					
OS	**5-year OS (95% CI)**				
	≤30 versus > 30	**88% (85–92)** versus **75% (65–86)**	94% (90–98) versus 84% (71–100)	85% (80–92) versus 68% (48–97)	82% (74–91) versus 70% (54–89)
	p value	***0.01***	*0.07*	*0.29*	*0.33*
	MVA HR (95% CI)				
	Continuous per 10	**1.14 (1.02, 1.27 *p = 0.01*)**	1.18 (0.96, 1.78 *p=0.12*)	1.20 (0.97, 1.49 *p=0.1*)	1.09 (0.92, 1.29 *p=0.31*)
	≤20 versus >20	1.39 (0.85, 2.26 *p=0.19*)	1.65 (0.61, 4.46 *p=0.33*)	1.41 (0.62, 3.21 *p=0.41*)	1.19 (0.56, 2.56 *p=0.65*)
	≤30 versus > 30	1.59 (0.92, 2.74 *p=0.09*)	2.31 (0.77, 6.9 *p=0.13*)	1.66 (0.32, 2.84 *p=0.32*)	1.26 (0.57–2.79 *p=0.57*)
CSS	**5-year CSS (95% CI)**				
	≤30 versus >30	90% (87–93) versus 86% (75–92)	97% (92–99) versus 90% (71–97)	87% (80–92) versus 68% (30–86)	86% (76–92) versus 94 (75–98)
	p value	*0.49*	*0.11*	*0.14*	*0.20*
	MVA HR (95% CI)				
	Continuous per 10	1.08 (0.92, 1.26 *p=0.34*)	1.27 (0.86, 1.88 *p=0.23*)	**1.27 (1.01, 1.58 *p = 0.03*)**	0.84 (0.66, 1.06 *p=0.14*)
	≤20 versus >20	1.26 (0.67, 2.37 *p=0.47*)	1.39 (0.33, 5.81 *p=0.65*)	1.78 (0.78, 4.05 *p=0.17*)	0.66 (0.23, 1.90 *p=0.45*)
	≤30 versus > 30	1.27 (0.58, 2.78 *p=0.55*)	3.48 (0.79, 15.33 *p=0.09*)	2.25 (0.77, 6.59 *p=0.14*)	0.29 (0.07, 1.19 *p=0.08*)

Abbreviations: MVA = multivariable analysis; TNM8 = 8th edition UICC/AJCC TNM staging criteria; CDDP-D = cisplatin cumulative dose; PYs = pack-years; OS = overall survival; CSS = cause-specific survival; HR = hazard ratio.
Note: MVA for OS was adjusted for age, stage and smoking PYs. MVA for CSS was adjusted for stage and smoking PYs. MVA for TNM8 subgroups includes CDDP-D and smoking PYs.
Note: Significant *p*-values are in bold.

Oliva M, Huang SH, Xu W, Su J, Hansen AR, Bratman SV., et al. Eur J Cancer. 2019 Sep;118:112-120.

Beyond clinic-pathological factors, other biomarkers can aid risk-stratification and guide treatment decisions. The evaluation of the tumor immune-contexture in HPV-related disease is particularly relevant in the era of immunotherapy(119, 120). Virus-related tumors are characterized by increased baseline tumor immunogenicity, increased immune infiltration and higher PD-L1 expression, and as such, they are postulated to be more responsive to immunotherapies, and particularly, to immune-checkpoint inhibitors(121, 122). When compared to HPV-unrelated HNSCC, HPV-related OPSCC has been demonstrated to have a less immunosuppressive tumor microenvironment, characterized by increased tumor infiltrating lymphocytes (TILs), a higher percentage of CD8+ T cells, elevated levels of interferon gamma (IFN-y), a decreased CD4+/CD8+ ratio, and fewer T-regulatory T-cells(123-125). A preexisting adaptive host immune response against viral and tumor-specific antigens can account for these results, and this response may then trigger the production of PD-L1 in immune cells. In fact, retrospective analysis revealed that, independent of stage, both high PD-L1 expression in immune cells (~5%) and CD8+ tumor infiltrating cells (~30%) were favorable prognostic markers in HPV-related tumors(126). PD-L1 expression

along with genomic and transcriptomic features such as tumor mutational burden, gene-expression immune signatures are demonstrated biomarkers of response to antiPD-L1 agents but also may predict prognosis in both HPV-related and unrelated OPSCC(127). A review on the topic was published by the PhD applicant(119). A table summarizing the evidence on the predictive role of these biomarkers and the role they play in HPV-related disease is provided below.

Table 5. Immune biomarkers in HNSCC

Immune biomarkers	Assay	Predictive value in HNSCC[a]		Evidence available
		HPV$^-$	HPV$^+$	
PD-L1 expression	PD-L1 staining by immunohistochemistry in tumor cells/immune cells (different cut-offs)	Positive[b]	Positive[b]	Prospective randomized clinical trials (Table 2).
Smoking	• Smokers versus nonsmokers • Smoking mutational signatures in tumor samples	Negative No data	Uncertain No data	Retrospective analysis of prospective trials [9]. Retrospective studies [67].
Tumor immune-cell infiltration	Presence of CD8$^+$ T cells PD-1$^+$ TIM-3$^+$ CD8$^+$ T cells PD-1+ LAG-3$^+$ CD8$^+$ T cells	Positive Negative Negative		Retrospective analysis of noncontrolled cohorts [73].
Circulating immune cells	PD-1$^+$ CD8$^+$ T cells FoxP3$^+$ Tregs	Negative Negative		Prospective analysis in a randomized clinical trial[102].
Tumor mutational burden	Number of somatic coding missense mutations. • Tumor samples • Blood samples	 Positive No data	 Uncertain No data	Retrospective analysis of prospective clinical trial [74, 75]. Retrospective analysis from a noncontrolled cohort [73].
T-cell-inflamed phenotype	Immune-related gene expression signatures	Positive	Positive	Retrospective analysis of prospective clinical trial [74, 75, 104]. Retrospective analysis from a noncontrolled cohort [73].
Microbiota	16S rRNA high throughput sequencing of saliva and stool	Oral microbiota: nonpredictive Intestinal microbiota: no data yet		Retrospective analysis of prospective randomized clinical trial [133].

[a]Predictive values in HPV$^-$ and HPV$^+$ subgroups were defined positive or negative if a statistically significant correlation between response and the immune biomarker was described in the referenced studies; uncertain if no significant correlation was found; no data if no studies had evaluated the role of the biomarker in this setting at the time of this publication.
[b]The positive correlation between PD-L1 expression and treatment response was not consistent across the studies.

Oliva M, Spreafico A, Taberna M, Alemany L, Coburn B, Mesia R Ann Oncol. 2019 Jan 1;30(1):57-67

1.11. Role of microbiome in head and neck squamous cell carcinoma

Head and neck cancers arise from the epithelium and mucosa of the oral and pharyngolaryngeal tract. These compartments are constantly exposed to external aggressions such as smoking, alcohol consumption, or infections, which can alter their microbiome composition(128, 129). Epstein–Barr virus (EBV) and human papillomavirus (HPV) are two well-established etiopathological agents of nasopharyngeal carcinoma and oropharyngeal squamous cell carcinomas

(OPSCC), respectively (90, 130). Beyond these specific pathogen–tumor type causality relationships, accumulating evidence suggested a potential role of bacterial oral and tumor-associated microbiome in the pathogenesis and prognosis of HNSCC.

Oral dysbiosis has been correlated with increased risk of developing HNSCC (131, 132). Several retrospective case–control studies have found differential microbiome composition in the saliva and mucosal and/or tumor tissues of patients with these tumor types when compared to healthy individuals, suggesting an implication in tumor initiation and development(133, 134). For instance, increased relative abundance of oral *Porphyromonas gingivalis*, a bacteria linked to periodontal disease, and *Fusobacterium nucleatum* may favor the initiation of oral SCC through the activation of immune evasion mechanisms and oncogenic pathways(135, 136). *Fusobacterium nucletum* inhibits β-catenin signaling and increases TLR4 activation of p21-activated kinase and cyclin D1, which together lead to increased inflammation and suppression of Natural Killer T-cell activities, ultimately promoting malignancy(137). Other oral commensal bacteria seem to be protective against HNSCC: both *Kingella* and *Corynebacterium* species, among others, which are functionally implicated in the biodegradation and/or metabolization of carcinogens from tobacco and/or alcohol (e.g., Acetaldehyde), have been linked with decreased risk of head and neck squamous cell carcinomas (HNSCC) – particularly laryngeal primaries - among smokers/alcohol consumers in a nested case–control study within a prospective cohort(138, 139). Despite the variation in the oral microbiome composition and the prevalence of genera and species among the retrospective series, *Fusobacterium, Capnocytophaga*, *Prevotella,* and *Peptostreptococcus*, are the most commonly reported (132)(**Table 6**).

Table 6. Oral microbiome composition linked to HNSCC

Flora	Technique	Notes
Bacillus, *Enterococcus*, *Parvimonas*, *Peptostreptococcus*, and *Slackia*	16S rDNA V4 sequencing of saliva samples	Increased in cases of malignancy when compared to oral potentially malignant disorders
Parvimonas	16S rDNA sequencing of paired normal and tumor resections	Concentration of *Parvimonas* positively correlated to T-stage
Peptostreptococcus, *Fusobacterium*, *Alloprevotella*, and *Capnocytophaga*	16S rRNA sequencing of salivary samples	More abundant when comparing the microbiome of cancer patients to the control patients
Fusobacterium nucleatum, *Pseudomonas aeruginosa*, and *Campylobacter*	16S rRNA V1-V3 s equencing of tissue samples	An overabundance of these microbiota were noted in tumor tissue when compared to healthy tissue
Fusobacterium nucleatum, *Capnocytophaga sputigena*, *Porphyromonas endodontalis*, and *Gemella haemolysans*	NGS of oral swabs	The relative concentration of *P. endodontalis*, *Gemella morbillorum*, and *G. haemolysans* related to increased depth of invaision
Schlegelella and *Methyloversatilis*	16S rRNA sequencing	Relative abundance of these organisms related to worse prognosis
Prevotella, Stomatobaculum, and *Bifidobacterium*	16S rRNA V1-V3 sequencing of salivary samples	With a relative loss of *Fusobacterium*
Capnocytophaga gingivalis, *Prevotella melaninogenica*, and *Streptococcus mitis*	NGS of salivary samples	Examiners were able to reliably predict the presence of malignancy based upon these organisms
Oribacterium	16S rRNA sequencing of oral rinse	Examiners were able to reliably predict the presence of oral cavity cancer and oropharyngeal cancers based on the presence of *Oribacterium*

Abbreviations: HNSCC, head and neck squamous cell cancer; NGS, next generation sequencing; PCR, polymerase chain reaction; T-stage, tumor stage; rDNA, recombinant deoxyribonucleic acid; rRNA, ribo ribonucleic acid.

Orlandi E, Iacovelli NA, Tombolini V, Rancati T, Polimeni A, De Cecco L Oral Oncol. 2019 Dec;99:104453.

Oral microbiome composition has also been correlated with clinico-pathological features and prognosis in HNSCC. Guerrero-Preston et al. found associations between specific oral bacteria, predominantly *Fusobacterium nucleatum* and *Lactobacillus* species in saliva from cases of oral and oropharyngeal cancers, and found differential composition of specific genera such as *Veillonella, Prevotella, Streptococcus* and *Gemella* according to stage and HPV status, mainly (140). *F. Nucleatum* has also been associated with lower recurrence rates in oral cancer (136). Other authors have described associations between oral bacteria and HNSCC location and histology, indicating that intercompartmental dysbiosis might lead to different tumor types or vice versa (141).

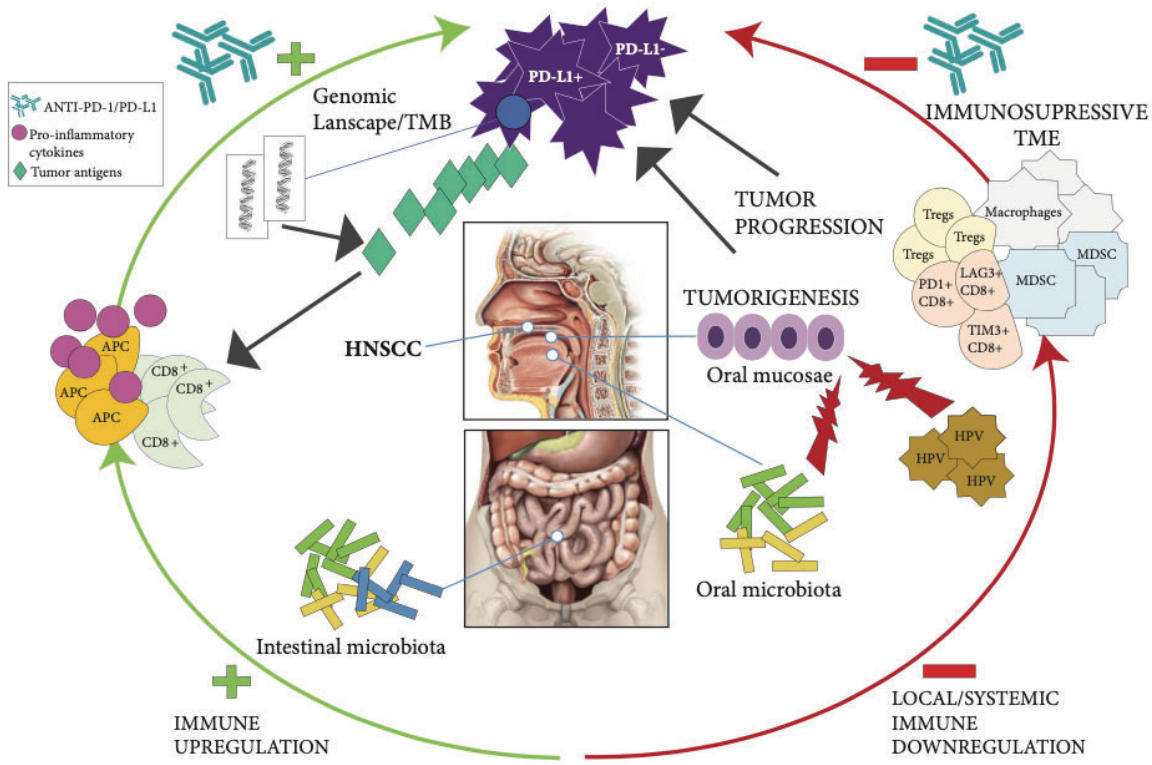
Fewer studies are available on the potential impact of tumor-associated microbiome on response and toxicity to anticancer therapies, all retrospective in nature. In terms of radiotherapy toxicity, oral dysbiosis has been correlated with increased oral mucositis (142, 143). Preclinical analysis in mice HNSCC models showed that oral inoculation with *P. gingivalis* led to high serum levels of the inflammatory cytokine IL-6 and that anti-inflammatory drug treatment increased chemosensitivity, suggesting that pathogen-induced inflammation may contribute to chemoresistance in HNSCC(135). The group of Guerrero-Preston and col.

revealed that the predominance of F*. nucleatum* was associated with down-regulation of immune-signaling pathways and upregulation of oncogenic Wnt/Beta-catenin pathways in HNSCC patients treated with surgery, chemoradiotherapy (CRT), and ICI (144), indicating the potential role of this bacteria in modulating response to treatment. In this regard, increased relative abundance of intratumoral *F. nucleatum* has been associated with poor response to curative-intent chemoradiation and higher risk of recurrence in esophageal and rectal cancers(14, 145). Given that chemotherapy agents are similar (ie. Platinum) and that *F. nucleatum* seems to be linked to HNSCC, evaluating the impact of these bacteria on treatment response and outcome should be pursued.

However, to date, there is no clinical evidence of oral/tumor-associated microbiome as a biomarker of response to standard therapies such as radiotherapy, chemotherapy, or immunotherapy in HNSCC. The only study that evaluated the oral microbiome in a subgroup of patients with recurrent/metastatic HNSCC treated with antiPD-1 agent nivolumab within the CheckMate-141 clinical trial failed to show any correlation with treatment response (146). However, the small number of patients and the low percentage of responses might have influenced these results.

In conclusion, there is currently no consensus in defining a diagnostic, prognostic and/or predictive HNSCC-associated oral/tumor/gut microbiome signatures given the differential results across studies. This is mainly due to the heterogeneity of the studies, mostly retrospective series and varying sample types (oral wash, tumor tissue sample, oral swab, etc.), stage, treatment history, and patient population, as well as the factors directly related with analysis methodology and patient-specific factors. The potential interactions between oral, tumor associated and intestinal microbiome in head and neck squamous carcinoma and their hypothesized role in modulating antitumor immunity and response to therapies are shown in **Figure 8.**

Figure 8. Interactions between the oral and intestinal microbiome, immune responses and HNSCC



Interactions between the oral and intestinal microbiome, immune responses and the HNSCC TME. The composition of the oral microbiota alters the oral mucosae contributing to tumor development and progression in the context of other coexisting factors such as HPV infection. Intestinal and oral microbial composition and diversity regulate systemic and local immune responses modulating the TME along with other immune biomarkers such as TMB or immune checkpoint protein expression, ultimately dampening or enhancing antitumor immune responses.

Oliva M, Spreafico A, Taberna M, Alemany L, Coburn B, Mesia R Ann Oncol. 2019 Jan 1;30(1):57-67

1.12. Annex: Supporting publications

The following publications are preliminary work led by or in which the applicant has actively participated that are relevant and related to the content of this thesis. The articles are exposed in chronological order of publication.

1. **Marc Oliva**, Anna Spreafico, Miren Taberna, Laia Alemany, Bryan Coburn, Ricard Mesia, Lillian L. Siu*. Immune biomarkers of response to immune-checkpoint inhibitors in head and neck squamous cell carcinoma.* Annals of Oncology (2019) 30(1): 57–67. (Review article). Q1 (D1); Impact Factor 18.2
2. **Marc Oliva,** Shao Hui Huang, Wei Xu, Jie Su, Aaron R Hansen, Scott V Bratman, Jolie Ringash, Raymond Jang, John Cho, Andrew Bayley, Andrew J Hope, Eric Chen, Meredith Giuliani, John Waldron, Ilan Weinreb, Bayardo Perez-Ordonez, Douglas Chepeha, John Kim, Brian O Sullivan, Lillian L Siu, Anna Spreafico*. Impact of cisplatin dose and smoking pack-years in human papillomavirus-positive oropharyngeal squamous cell carcinoma treated with chemoradiotherapy.* European Journal of Cancer. (2019) Sep:118:112-120. (Original Article). Q1; Impact Factor 8.4
3. Daniel V. Araujo, Geoffrey A. Watson, **Marc Oliva,** Alya Heirali, Bryan Coburn, Anna Spreafico, Lillian L. Siu*. Bugs as drugs: The role of microbiome in cancer focusing* on immunotherapeutics. Cancer Treatment Reviews 92 (2021) 102125. (Review article). Q1(D1); Impact Factor 11.8
4. **Marc Oliva**, Nuria Mulet-Margalef, Maria Ochoa-De-Olza, Stefania Napoli, Joan Mas, Berta Laquente, Laia Alemany, Eric J. Duell, Paolo Nuciforo and Victor Moreno. *Tumor-Associated Microbiome: Where Do We Stand?* Int. J. Mol. Sci. (2021) 22, 1446. (Review article). Q2; Impact Factor 5.9
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REVIEW

Immune biomarkers of response to immune-checkpoint inhibitors in head and neck squamous cell carcinoma

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Anti-programmed cell death protein 1 (PD-1) agents have become the standard of care for platinum-refractory recurrent/metastatic head and neck squamous cell carcinoma (HNSCC) and are currently being evaluated in various disease settings. However, despite the gain in overall survival seen in some of the clinical trials, the majority of patients display primary resistance and do not benefit from these agents. Taking into consideration the potentially severe immune-related toxicities and their high cost, the search for predictive biomarkers of response is crucial. Besides Programmed death ligand-1 (PD-L1) expression, other biomarkers such as immune infiltration, tumor mutational burden or immune-gene expression profiling have been explored, but none of them has been validated in this disease. Among these, the microbiota has recently garnered tremendous interest since it has proven to influence the efficacy of PD-1 blockade in some tumor types. With the accumulating evidence on the effect of the microbiota in HNSCC tumorigenesis and progression, the study of its potential role as a predictive immune biomarker is warranted. This review examines the available evidence on emerging immune predictive biomarkers of response to anti-PD-1/PD-L1 therapy in HNSCC, introducing the microbiota and its potential use as a predictive immune biomarker in this disease.

Key words: head and neck squamous cell carcinoma, immune checkpoint inhibitors, anti-PD-1/PD-L1, biomarkers, microbiota

Introduction

Immune-checkpoint inhibitors (ICI) targeting cytotoxic T-lymphocyte antigen 4 and programmed cell death protein-1 (PD-1) and its ligands, programmed death ligand-1 (PD-L1)/2, have shown a significant and consistent benefit in survival when compared with standard therapies in prospective randomized clinical trials, leading to their regulatory approval in multiple tumor types [1–5]. In head and neck squamous cell carcinoma (HNSCC), anti-PD-1 antibodies are the first immunotherapeutic agents to demonstrate evidence of response durability and survival benefit in platinum-pretreated recurrent and metastatic (R/M) disease [6–9]. However, despite the encouraging results which led to the approval of nivolumab and accelerated approval of pembrolizumab by the US Food and Drug Administration (FDA) for platinum-refractory R/M HNSCC, the overall response rates (ORRs) of these agents ranged from only ∼13%–18% [9, 10].

Up to 60% of patients across different tumor types, including HNSCC, display primary resistance to anti-PD-1/PD-L1 agents [11]. Several mechanisms have been suggested such as poor tumor immunogenicity, limited intratumoral immune cell infiltration, coexpression of multiple inhibitory receptors, and induction of immunosuppressive pathways within the tumor microenvironment (TME) [12–14]. To overcome this resistance,

Table 1. Emerging immune biomarkers of response to anti-PD-1/PD-L1 agents in HNSCC

Immune biomarkers	Assay	Predictive value in HNSCC[a]		Evidence available
		HPV$^-$	HPV$^+$	
PD-L1 expression	PD-L1 staining by immunohistochemistry in tumor cells/immune cells (different cut-offs)	Positive[b]	Positive[b]	Prospective randomized clinical trials (Table 2).
Smoking	• Smokers versus nonsmokers • Smoking mutational signatures in tumor samples	Negative No data	Uncertain No data	Retrospective analysis of prospective trials [9]. Retrospective studies [67].
Tumor immune-cell infiltration	Presence of CD8$^+$ T cells PD-1$^+$ TIM-3$^+$ CD8$^+$ T cells PD-1+ LAG-3$^+$ CD8$^+$ T cells	Positive Negative Negative		Retrospective analysis of noncontrolled cohorts [73].
Circulating immune cells	PD-1$^+$ CD8$^+$ T cells FoxP3$^+$ Tregs	Negative Negative		Prospective analysis in a randomized clinical trial[102].
Tumor mutational burden	Number of somatic coding missense mutations. • Tumor samples • Blood samples	 Positive No data	 Uncertain No data	Retrospective analysis of prospective clinical trial [74, 75]. Retrospective analysis from a noncontrolled cohort [73].
T-cell-inflamed phenotype	Immune-related gene expression signatures	Positive	Positive	Retrospective analysis of prospective clinical trial [74, 75, 104]. Retrospective analysis from a noncontrolled cohort [73].
Microbiota	16S rRNA high throughput sequencing of saliva and stool	Oral microbiota: nonpredictive Intestinal microbiota: no data yet		Retrospective analysis of prospective randomized clinical trial [133].

[a]Predictive values in HPV$^-$ and HPV$^+$ subgroups were defined positive or negative if a statistically significant correlation between response and the immune biomarker was described in the referenced studies; uncertain if no significant correlation was found; no data if no studies had evaluated the role of the biomarker in this setting at the time of this publication.
[b]The positive correlation between PD-L1 expression and treatment response was not consistent across the studies.

many ongoing clinical trials are evaluating combination strategies with other immunotherapies, targeted agents, chemotherapy and radiotherapy, not only in R/M HNSCC, but also in the locoregionally advanced setting (*NCT02952586*, *NCT03040999*) [15]. This is of particular relevance as a proportion of patients with R/M HNSCC might experience rapid progression and decreased survival when treated with single-agent anti-PD-1/PD-L1 [16].

However, the potential immune-related toxicities of ICI and their high cost have urged the search for prospectively validated predictive biomarkers of response including PD-L1 protein expression, intratumoral immune cell infiltration, immune-gene expression profiling, and tumor mutational burden (TMB) [13, 14, 17]. Specifically, in HNSCC, none of them have been validated and ongoing exploration continues [9, 18].

Recently, the immunomodulatory role of the gut microbiota, defined as the collective microorganisms inhabiting the gastrointestinal tract, has raised a special interest, since its composition has proven to influence anti-PD-1 efficacy in preclinical models and has been associated with treatment responsiveness in patients with melanoma and some epithelial-derived tumors [19–22]. Interestingly, many retrospective studies in HNSCC have suggested that the oral microbiota might also be crucial for tumor development and progression, treatment-related toxicity and disease recurrence [23–25].

This review examines the available evidence on emerging immune predictive biomarkers of response to ICI in HNSCC, introducing the microbiota and its potential use as a predictive immune biomarker in this disease (Table 1).

Overview of emerging immune biomarkers in HNSCC

Is PD-L1 expression a reliable biomarker of response in HNSCC?

PD-L1$^+$ tumors in general tend to demonstrate improved response rates to anti-PD-1/PD-L1 therapies, in comparison to PD-L1$^-$ tumors [26]. This correlation has been consistent with different anti-PD-1/PD-L1 drugs across many tumor types [5, 27, 28]. Most clinical trials evaluating ICI in R/M HNSCC suggested a similar pattern [29–31], and data from phase III randomized trials investigating pembrolizumab in the R/M setting

Table 2. Anti-PD-1/PD-L1 agents tested in R/M HNSCC [6–9, 29–31, 33, 34, 52, 134]

Agents	Target	Phase/study	*N*	PD-L1 expression Location	Cut-off	ORR (%)			OS (HR)[a]		
						Overall	PD-L1$^+$	PD-L1-	Overall	PD-L1$^+$	PD-L1$^-$
Nivolumab	PD-1	III (CHECKMATE-141)	240	TCs	>1%	13.3%	17%	11.8%	0.68	0.55	0.73
Pembrolizumab	PD-1	I (KEYNOTE-012)	132	TCs+ICs	>1%	18%	22 %	4%	NA	NA	NA
				TCs only	>1%		17 %	7%			
		III (KEYNOTE-040)	247	TCs+ICs(CPS)	CPS > 1%	14.6%	17.3%	Ø	0.80 (*P* 0.016)	0.74 (*P* 0.0049)	Ø
				TCs (TPS)	TPS > 50%		26.6%	Ø		0.53 (*P* 0.0014)	Ø
		III (KEYNOTE-048)	882	TCs+ ICs (CPS)	CPS>1	Ø	19.1%	Ø	Ø	0.78 (*P* 0.0086	Ø
					CPS>20		23.3%	Ø		0.61 (*P* 0.0007)	Ø
Durvalumab	PD-L1	I (MEDI4736-1108)	62	TCs	>25%	10%	18%	8%	NA		NA
		II (HAWK)	112	TCs	>25%	NA	16.2%	NA	NA		
		II (CONDOR)	67	TCs	<25%	NA	NA	6%	0.99 (*P* 0.89)		
Atezolizumab	PD-L1	I (GO27831)	32	ICs	IC2/3: >5% IC0/1: <5%	22%	24%	14%	NA		NA

[a]HR for OS resulting from: nivolumab and pembrolizumab versus investigator's choice of chemotherapy (Docetaxel, Methotrexate and Cetuximab) in the CHECKMATE-141 and KEYNOTE-040 studies, respectively; pembrolizumab monotherapy versus EXTREME regimen in the KEYNOTE-048 study; durvalumab versus tremelimumab plus durvalumab in the CONDOR study.

ORR, overall response rate; OS, overall survival; HR, hazard ratio; TCs, tumor cells; ICs, immune cells; CPS, number of PD-L1-positive cells (tumor cells, lymphocytes, macrophages) divided by total number of tumor cells × 100; TPS, percentage of tumor cells with membranous PD-L1 expression; NA, not applicable; Ø, no data available.

(KEYNOTE-040 and KEYNOTE-048) endorsed this trend by demonstrating significantly increased survival in PD-L1$^+$ patients [8, 32, 33]. However, CHECKMATE-141 failed to show a significant correlation between PD-L1 expression and tumor response or survival when evaluating nivolumab in the platinum-refractory R/M setting [9, 34] (Table 2).

The discordance of the results across studies might be explained by several reasons. One of the most relevant is the lack of uniformity in the assays and the variability in the thresholds used to define PD-L1 positivity, which have led to the launch of harmonization projects on PD-L1 assays by the scientific community and regulatory agencies [28, 35, 36]. This inconsistency is evident in the development of anti-PD-1/PD-L1 agents investigated to date in R/M HNSCC, including pembrolizumab, nivolumab, atezolizumab, durvalumab and avelumab, thus impairing cross-study comparisons and undermining the value of PD-L1 as a biomarker [6, 9, 30–32, 37, 38]. Importantly, PD-L1 expression seems to be regulated by multiple signaling pathways, including MAPK, PI3K and Akt/PKB that are commonly altered in HNSCC [39–41]. As a consequence of these molecular crosstalks, PD-L1 is a dynamic biomarker that is subject to temporal variations and spatial heterogeneity. Its expression may change from the point of initial diagnosis to recurrence or progression, and may differ between primary and coexisting metastatic lesions [42–45]. Published reports on the intratumoral heterogeneity of PD-L1 expression in HNSCC demonstrate conflicting results [46, 47].

In HNSCC, PD-L1 is highly expressed not only by tumor cells, but also by immune cells present in the TME, including regulatory T cells (Tregs), natural killer (NK) cells and antigen presenting cells (APCs) [18, 48–51]. Across various cancer types, it remains unclear whether PD-L1 expression and thresholds should take into consideration all or only selected cell populations. Both pembrolizumab and atezolizumab used combined scores based on the ratio between tumor cells and immune cells expressing PD-L1 to define tumor PD-L1 positivity, and pembrolizumab did show a positive correlation with response and survival in the phase III KEYNOTE-040 study when using the combined positive score (CPS) [52]. Recently, the results from the phase III KEYNOTE-048 study in first line R/M HNSCC revealed that pembrolizumab monotherapy improved OS when compared with the EXTREME regimen in patients whose tumors had PD-L1 expression ≥1% and ≥20% by CPS [hazard ratio (HR) 0.78 (0.64–0.96), $P = 0.0086$ and HR 0.61 (0.45–0.83), $P = 0007$, respectively] [33]. However, in KEYNOTE-040, the correlation with clinical outcome was also strongly positive when using PD-L1 expression in tumor cells only (TPS ≥ 50%), congruent with the experience in non-small-cell lung cancer (NSCLC) in KEYNOTE-010 [53, 54]. In contrast, there was no correlation in the nivolumab CHECKMATE-141 study where PD-L1 expression was exclusively determined in tumor cells, although the thresholds used were different (>1%, 5% and 10%) [9]. These divergent results and the limited data available suggest no firm conclusion can be made in this regard, although CPS seems to be more predictive than TPS in HNSCC, and the required cut-off for the latter appears to be higher in the mentioned studies.

Nonetheless, it is noteworthy that, although relevant in a smaller percentage, PD-L1$^-$ tumors also benefit from ICI [9].

Therefore, additional factors beyond PD-L1 expression, such as human papillomavirus (HPV) status, tumor immune infiltration or TMB, might also contribute to treatment response.

Are HPV$^+$ tumors more responsive to immunotherapy?

HPV$^+$ oropharyngeal squamous cell carcinoma (OPSCC) is a biologically distinct disease with better prognosis and improved treatment responsiveness when compared with HPV$^-$ disease at the same or similar stage [55–57]. Virus-related tumor types are postulated to be more responsive to ICI due to intrinsic characteristics including baseline tumor immunogenicity, increased immune infiltration and increased PD-L1 expression [58, 59]. HPV$^+$ OPSCC have been shown to have a less immunosuppressive TME when compared with HPV$^-$ HNSCC, as it harbors greater infiltration by tumor infiltrating lymphocytes (TILs), higher proportion of CD8$^+$ T cells, increased levels of interferon gamma (IFN-γ), decreased CD4$^+$/CD8$^+$ ratio, and lower numbers of Tregs [60–64]. These findings can be explained by a preexisting adaptive host immune response against viral and tumor-specific antigens, which may in turn lead to PD-L1 expression in immune cells. Indeed, a recent retrospective study showed that not only CD8$^+$ TILs (≥30%) but also high PD-L1 expression in immune cells (≥5%) were both favorable prognostic factors in HPV$^+$ disease regardless of stage [65, 66].

Altogether these findings suggest a potentially higher sensitivity of HPV$^+$ disease to immune-checkpoint blockade. This hypothesis was initially supported by the results from the HNSCC cohort of the multibasket phase I KEYNOTE-012 trial in which HPV$^+$ tumors had increased ORR to pembrolizumab compared with those that were HPV$^-$ (25%–32% versus 14%) [6, 7]. However, these results were not reproduced in the phase III KEYNOTE-040 trial, and further studies investigating other anti-PD-1/PD-L1 agents have reported mixed results. For instance, increased response rates were observed among HPV$^+$ patients treated with durvalumab while no differences were seen with atezolizumab [30, 31]. In the CHECKMATE-141 study, nivolumab did not yield significant differences in ORR or OS between HPV$^+$ and HPV$^-$ patients [HR for OS 0.60 (0.37–0.97) versus 0.59 (0.38–0.92), respectively] [9, 32, 34].

The inconsistencies in the abovementioned trials might be explained by other coexisting factors beyond PD-L1 expression and immune infiltration. Smoking, mutational signatures and TMB are thought to influence response to ICI in HNSCC although their relevance differs between HPV$^+$ and HPV$^-$ disease (Table 1).

Smoking seems to contribute to a more immunosuppressive TME and negatively impact on anti-PD-1/PD-L1 efficacy in HNSCC. In CHECKMATE-141 study, the subgroup analysis reported a trend toward decreased survival benefit from nivolumab among smokers when compared with nonsmokers [9]. Similarly, a retrospective analysis of 81 HNSCC patients treated with anti-PD-1/PD-L1 showed that former/current smokers were less responsive to these agents when compared with never smokers. However, this correlation only remained significant among HPV$^-$ patients, suggesting the immunosuppressive effects of smoking may not be as significant in HPV$^+$ tumors [67]. In support of this, a genomic analysis of 287 HNSCC tumor samples revealed that smoking history and tumors with high smoking mutational signatures were correlated with decreased immune infiltration and downregulation of immune-signaling pathways in HPV$^-$ but not HPV$^+$ tumors [67].

Conversely, the presence of other mutational signatures unrelated to smoking such as APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) is of particular relevance in HPV$^+$ disease. Reduced exposure to exogenous carcinogens such as tobacco seems to favor the emergence of tumors with APOBEC-driven mutations such as *PI3KCA* [68, 69]. Moreover, APOBEC activity is known to be crucial for innate and adaptive immune responses, and HPV infection is thought to enhance it in an attempted host immune response against the virus. In a study analyzing over 500 HNSCC tumor samples, APOBEC mutational signatures were associated with upregulation of immune-signaling pathways [69]. APOBEC-driven mutagenesis might alter tumor immunogenicity in HPV$^+$ disease impacting on immune checkpoint efficacy. Parenthetically, the presence of APOBEC signatures has been associated with increased immune infiltration and PD-L1 expression in other tumor types [70–72].

Increased TMB and neoantigen load have been shown to correlate with response to ICI in HPV$^-$ HNSCC, whereas most of the studies conducted to date have refuted their predictive value in HPV$^+$ patients [73–75]. TMB is a quantitative measure of the total number of coding mutations in the tumor genome. Theoretically, the higher the number of missense mutations, the higher expression of tumor neoantigens which can elicit the greatest antitumor immune response and increase sensitivity to ICI. A retrospective analysis from KEYNOTE-012 and -055 demonstrated a stronger correlation between response to pembrolizumab and high TMB and neoantigen load in the HPV$^-$ subgroup than HPV$^+$ subgroup [75]. As a matter of fact, in virally induced tumors such as HPV$^+$ tumors or Merkel-cell carcinoma, response rates to ICI are higher than expected when adjusted for TMB and compared with other tumors types, suggesting immune responses may also be triggered by virus-specific antigens rather than by tumor-neoantigens alone [39, 76–78]. In support of this, a retrospective study analyzing a cohort of 126 patients with R/M HNSCC treated with anti-PD-1/PDL-1 agents showed that HPV$^+$ patients had, as expected, lower TMB (8.2 versus 4.7 mut/MB, $P < 0.01$) when compared with HPV$^-$ disease, while the number of responses was similar (7 versus 10 responses, $P = 0.54$) [73]. More importantly, among HPV$^+$ patients, responders had increased CD8$^+$ TILs regardless of TMB.

Overall, with the current available data, it is not possible to determine whether HPV$^+$ OPSCC have higher (or lower) sensitivity to ICI when compared with HPV$^-$ disease. HPV positivity alone does not seem to be a reliable biomarker of response to ICI and needs to be interpreted along with other companion clinical and molecular biomarkers.

Is there a role for tumor immune infiltration and T-cell-inflamed phenotypes?

Tumor immune infiltration implies initial recognition by the immune system and might indicate an antitumor immune response [79]. Multiple immune cells coexist within the TME, including TILs (CD8$^+$ T cells and Tregs), NK cells, macrophages, APC and myeloid-derived suppressor cells. The composition of these

immune cells within TME, recently defined as immune contexture, has prognostic implications but can also be predictive of response to therapies [17, 61, 80]. For instance, $CD8^+$ T-cell infiltration at baseline has been correlated with increased response to anti-PD-1/PD-L1 agents in melanoma [81, 82].

HNSCC tumors are highly immune-infiltrated but overall characterized by an immunosuppressive TME [48, 83]. Many retrospective studies have attempted to assess the prognostic and predictive value of tumor immune cell infiltration (supplementary Table S1, available at *Annals of Oncology* online) [18, 62, 63, 84–89]. Despite the heterogeneity of these studies, increased infiltration by $CD8^+$ T cells is the only immune cell type in HNSCC consistently proven to be correlated with increased survival regardless of tumor location, stage and treatment [61, 65]. A retrospective evaluation of 126 patients diagnosed with R/M HNSCC treated with anti-PD-1/PD-L1 agents showed that increased tumoral infiltration by $CD8^+$ T cells and an increased ratio $CD8^+$ T cells/Tregs were positively correlated with treatment response, indicating their potential role as predictive biomarkers [73].

In addition, the relative proportion of the various immune cell subsets and their location within the TME may be of relevance in predicting response to ICI. The immunoscore (IS) is a tool quantifying the density of $CD8^+$ T cells within the tumor center versus the invasive margin. Increased number of $CD8^+$ T cells in the tumor center (high IS) is thought to indicate an effective antitumor immune response and has been proven to be an independent prognostic biomarker in early stage colorectal cancer, melanoma and NSCLC [80, 90–92]. In HNSCC, a high IS is associated with lower levels of Tregs, increased PD-L1 and MHC type I expressions in tumor cells [62, 93], suggesting its potential to identify a subset of tumors with increased sensitivity to anti-PD-1/PD-L1 therapy. However, the predictive role of IS in HNSCC has not been explored yet.

The coexpression of other inhibitory immune-checkpoint molecules such as TIM-3 (T-cell immunoglobulin and mucin domain-containing protein 3), lymphocyte-activating gene 3 (LAG-3) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) has also shown to impair immune T-cell-mediated responses, conferring resistance to anti-PD-1/PD-L1 agents in preclinical models and in patients across different tumor types such as melanoma and NSCLC [15, 94–99]. In HNSCC, a recent study showed intratumoral exhausted $PD\text{-}1^+$ $CD8^+$ T cells expressing TIM-3 or LAG-3 were higher among nonresponders to anti-PD-1 therapy [73]. In this regard, the predictive value of response to ICIs offered by immunophenotyping of circulating T-cell subsets versus TILs has demonstrated relevance in melanoma and NSCLC but it is still unknown in HNSCC [100, 101]. In a substudy of CHECKMATE-141 evaluating treatment with nivolumab beyond progression, responders had significantly lower levels of circulating $PD\text{-}1^+$ $CD8^+$ T cells at baseline and lower levels of $PD\text{-}1^+$ Tregs at day 43, indicating circulating exhausted T cells could be a negative predictive biomarker to anti-PD-1/PD-L1 agents [102]. Although the available data are still limited and should be interpreted with caution, determining the coexpression of inhibitory checkpoint molecules in intratumoral and/or circulating T-cell subsets could be predictive of resistance to anti-PD-1/PD-L1 agents and potentially indicate the need for ICI combinations in selected cases of HNSCC.

Gene-expression profiling (GEP) signatures that identify tumors with a T-cell-inflamed phenotype have shown promising results in predicting response to anti-PD-1/PD-L1 agents [103, 104]. A 18-gene T-cell-inflamed signature including genes that reflect an ongoing adaptive Th1 and cytotoxic $CD8^+$ T-cell response (including IFN-γ signaling, cytolytic activity, antigen presentation and T cell trafficking) has been tested in two HNSCC cohorts from prospective clinical trials (KEYNOTE-012 and KEYNOTE-055) treated with single-agent pembrolizumab showing a positive correlation with response and survival, regardless of HPV status [74, 75]. This signature has been recently validated in additional tumor cohorts from KEYNOTE-012 and -028 studies, including melanoma and HNSCC. The study confirmed its predictive value as a biomarker of response to pembrolizumab and also revealed a positive correlation with PD-L1 expression by CPS [105].

Despite the prognostic implications and early data suggesting a correlation between TILs and response to anti-PD-1/PD-L1 therapy, prospective validation is needed. Moreover, identifying a T-cell-inflamed phenotype and determining coexisting immune cells and coexpression of other inhibitory immune checkpoint molecules beyond PD-1/PD-L1 within the TME could be instrumental to differentiate tumors that will likely be responsive to anti-PD-1/PD-L1 antibodies as single agents from those that may benefit from combined ICI for efficacy.

Tumor mutational burden and HNSCC mutational landscape

TMB has been recently evaluated as a potential biomarker of response to immune checkpoint blockade in prospective clinical trials and across many tumor types [77, 106–109]. An initial retrospective analysis of 27 tumor types and subtypes among patients who received PD-1/PD-L1 inhibitors demonstrated a significant correlation between TMB and response rate to these agents [77]. In this study, TMB was reported as a median number of coding somatic mutations per megabase (*N* mut/MB). Melanoma and squamous cell carcinoma of the skin (15–50 mut/MB) followed by tobacco-related cancers including NSCLC, urothelial cell carcinoma and HNSCC (5–10 mut/MB) comprised malignancies with the highest TMB [77]. Retrospective subset analyses of clinical trials evaluating pembrolizumab, atezolizumab and nivolumab in metastatic melanoma, NSCLC, urothelial carcinoma and HNSCC have demonstrated not only increased ORR but also improved survival in patients with high TMB [75, 106–108, 110]. These results were consistent across the studies, tumor type and anti-PD-1/PD-L1 agents. However, the cut-off and measure used to define a high TMB differed between studies, thus precluding direct comparisons. These results were further supported by a retrospective analysis of 126 HNSCC patients treated with anti-PD-1/PD-L1 agents. TMB was found to be significantly higher among responders (21.3 versus 8.2 mut/MB, $P < 0.01$) and was correlated with increased median OS (20 months if TMB > 10 mut/MB versus 6 months if TMB < 5 mut/MB, $P = 0.01$) in HPV^- disease [73]. A combined biomarker analysis of multiple studies evaluating the correlation between TMB, T-cell-inflamed GEP, PD-L1 expression by CPS and response to pembrolizumab in HNSCC showed no

significant correlation between TMB and inflammatory biomarkers (i.e. GEP or PD-L1). While this analysis did not stratify by HPV status, it suggests TMB and inflammatory biomarkers have distinct and independent predictive values, and may be used orthogonally to identify responders to pembrolizumab [105].

In addition to TMB, the specific tumor mutational landscape might be of biological relevance. Tumors characterized by mutations affecting DNA damage response, such as those with microsatellite instability high (MSI-H) or mismatch repair deficiency (dMMR), have the highest mutational load [77, 111]. These tumors have been shown to be particularly sensitive to ICI in prospective clinical trials, leading to the FDA approval of pembrolizumab for patients with dMMR or MSI-H tumors, regardless of histology [112, 113]. The estimated incidence of MSI-H tumors among HNSCC has been reported to be about 8% [114]. However, a recent study identified a subgroup of HNSCC responders to anti-PD-1/PD-L1 whose tumors were enriched with somatic mutations derived from frameshift events in tumor suppression genes such as *NOTCH* and *SMARCA4* [73]. These cases are similar to what has been described in tumors with dMMR, with baseline increased mutational burden and greater sensitivity to ICI. The authors suggested this finding might represent a novel mutational signature in HNSCC with potential predictive value, although further validation is warranted.

HNSCC genomic classification described by the TGCA might be considered as well [39]. Four subtypes have been defined on the basis of gene expression: atypical, mesenchymal, basal and classical. The mesenchymal subtype, e.g. characterized by alterations in genes related to innate immunity, downregulation of MHC type I expression and deficient antigen-presentation machinery, would unlikely respond to anti-PD-1/PD-L1 agents.

Overall, while the predictive role of the specific molecular subtypes is yet to be explored, TMB has shown promising results and might become a useful predictive biomarker of immune-checkpoint blockade efficacy in HNSCC. However, similar to what occurred with the PD-L1 assay, the lack of uniformity in the methods used to determine the mutational burden (e.g. measured in the tumor or in the blood) and the variability of the thresholds used across studies are hampering the interpretation and extrapolation of the results obtained. Thus, standardization should be pursued when designing biomarker-validating studies using TMB. Moreover, TMB has not shown to correlate with PD-L1 expression or GEP signatures [73, 75, 105], again indicating the interactions between the tumor, TME and the immune system are complex and dynamic.

Introducing the microbiota as a potential immune biomarker for HNSCC

The microbiota in head and neck cancer

The composition of the microbiota present in the orogastrointestinal tract has been associated with immune dysregulation and initiation and progression of many cancers [23, 115–118]. The precise mechanisms of these associations are not known, but compositional and functional changes in the microbiota can induce or exacerbate chronic inflammation, resulting in cell damage and alteration of local and systemic immune homeostasis, which may affect local and distant carcinogenesis, ultimately dampening or enhancing antitumor immune responses [116, 119]. HNSCC arise from an epithelium and mucosae located in the oral cavity and the pharynx; both sites are constantly exposed to environmental factors that can alter the oral microbiota [120, 121]. Retrospective cohort studies have shown different microbiota composition in the saliva of HNSCC patients compared with healthy controls, while the presence of specific bacteria has been associated with reduced risk of developing HNSCC [23, 122–124]. Moreover, differentially enriched microbiota found in HPV^+ and HPV^- OPSCC and oral cavity SCC indicates the existence of specific microbiota according to tumor location and HPV status [24]. Nonetheless, some authors have underlined the challenge of distinguishing whether the changes observed in the oral microbiota from HNSCC patients are influenced by the TME and/or by local and systemic cancer therapies, since most of the studies to date have retrospectively evaluated small, heterogeneous and noncontrolled cohorts of patients comprising different tumor sites, variable disease stages, and treatment with multiple modalities [23]. In this regard, a study analyzing the oral microbiota present in the saliva of HNSCC patients before and after treatment [including surgery, chemoradiotherapy (CRT) and ICI] showed an association between specific oral bacteria composition (*Fusobacterium* and *Lactobacillus*), down-regulation of immune-signaling pathways and upregulation of oncogenic Wnt/Beta-catenin pathways [125]. Altogether these findings suggest that the oral microbiota might represent a promising prognostic and predictive biomarker in this disease (Figure 1).

Exploiting the microbiota as a biomarker of response to immunotherapy

Accumulating evidence has implicated that intestinal microbiota can modulate host anticancer immune responses and alter the efficacy of anticancer therapies, including immunotherapy [19, 126–131]. Two preclinical studies using mouse models of melanoma and lung cancer revealed a correlation between the presence of specific commensal intestinal bacteria (*Bifidobacterium*) and response to ICI [20, 132]. This was further supported by two recent publications evaluating the gut microbiome in patients with melanoma and epithelial-derived tumors, showing improved anti-PD-1/PD-L1 efficacy among patients harboring specific intestinal bacteria (the species of *Akkermansia muciniphila* and members of the *Ruminococcaceae* family) and higher microbial diversity [21, 22]. Remarkably, these microbiota were also correlated with enhanced local and systemic immune response, reduction in tumor growth and restoration of response to anti-PD-1/PD-L1 therapy in germ-free mice transplanted with fecal microbiota from responding patients. These latter findings indicate the potential modulation of the microbiota as a viable therapeutic target to increase response to ICI.

Whether the microbiota has a role in predicting response to immunotherapy in HNSCC is yet to be determined. Only one substudy from CHECKMATE-141 explored the role of the oral microbiota measured in the saliva as a predictive biomarker in patients with R/M HNSCC treated with nivolumab, showing no

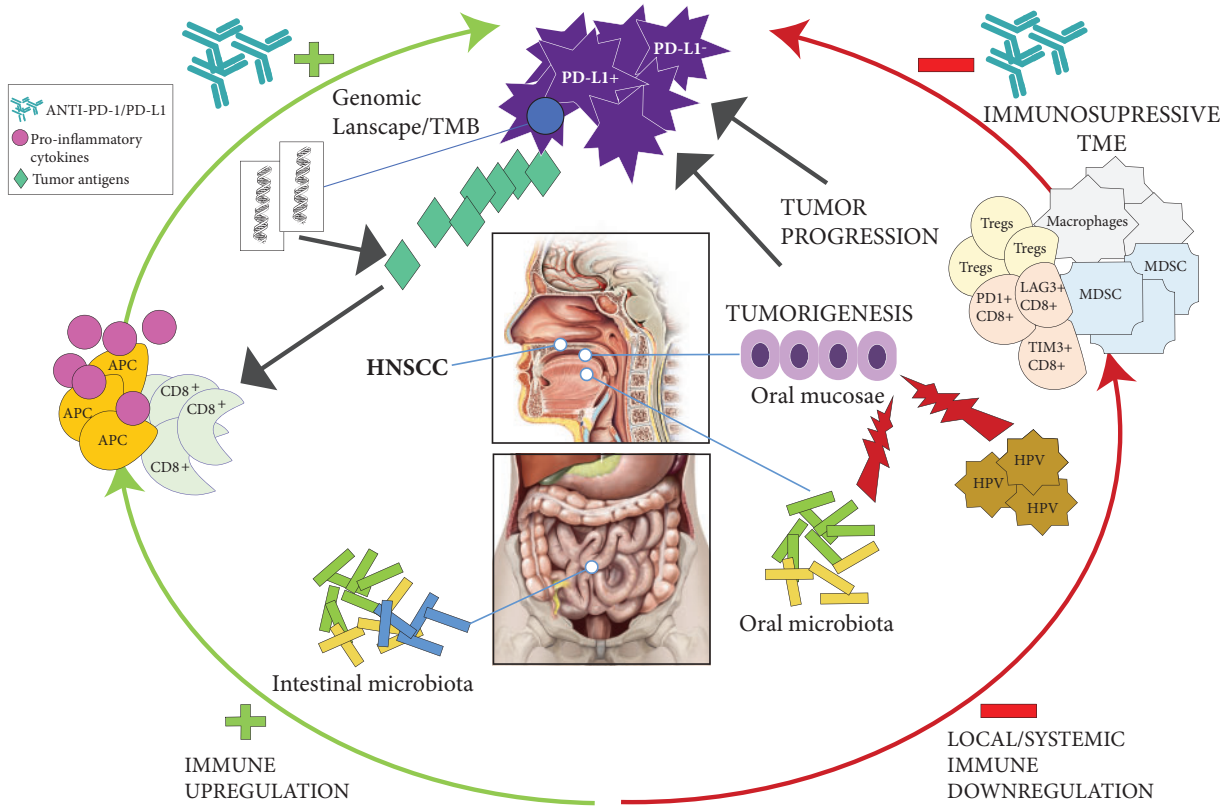

Figure 1. Interactions between the oral and intestinal microbiome, immune responses and the HNSCC TME. The composition of the oral microbiota alters the oral mucosae contributing to tumor development and progression in the context of other coexisting factors such as HPV infection. Intestinal and oral microbial composition and diversity regulate systemic and local immune responses modulating the TME along with other immune biomarkers such as TMB or immune checkpoint protein expression, ultimately dampening or enhancing antitumor immune responses.

significant correlation with treatment efficacy or survival [9, 133]. However, the study had several limitations, including the lack of uniformity in sample collection, the small number of responses for correlation and importantly, the omission of intestinal microbiota. The predictive role of the oral microbiota was also investigated in melanoma patients treated with anti-PD-1/PD-L1 therapy, again reporting no association with treatment outcome, in contrast to the positive correlation observed with the intestinal microbiota composition [22]. Differential bacterial composition between these anatomical sites suggests oral and intestinal microbiota likely represent distinct entities with specific disease associations.

Considering the immunomodulatory effects of the intestinal microbiota and the growing evidence of the oral microbiota impacting HNSCC tumorigenesis and progression, the study of their role as a predictive biomarker of response to ICI in this disease is warranted. Hence, our group is currently conducting a research study at the Princess Margaret Cancer Centre to prospectively evaluate the oral and intestinal microbiota in a homogeneous cohort of patients diagnosed with locoregionally advanced OPSCC treated with definitive chemoradiotherapy. The overarching goal of this project is to characterize and explore the correlation with both oral and intestinal microbiota measured in the saliva and stool, respectively, by using 16S rRNA sequencing, in order to obtain a deeper understanding of their relationship with treatment response. The results of this ongoing study will serve as a fundamental basis to evaluate oral and intestinal microbiota signatures and their role as predictors of response to ICI in patients treated within the CCTG HN.9 clinical trial, a multicenter phase II noncomparative randomized study evaluating ICI plus RT followed by maintenance ICI versus standard chemoradiotherapy in intermediate-risk, HPV^{+} locoregionally advanced OPSCC (NCT034106615).

Discussion

Conclusion

Anti-PD-1 agents have become the standard of care for the platinum-refractory R/M HNSCC. Results from clinical trials evaluating their role in additional disease settings are pending, but clearly such compounds are already an important therapeutic backbone in this malignancy. As such, appropriate selection of patients who will benefit from these therapies is crucial. To date, there are no validated predictive biomarkers of response that are applicable uniformly to all HNSCC patients, although many candidate biomarkers with promising results are undergoing investigations. A systematic computational analysis of all clinically

annotated biomarker data would be invaluable to further the knowledge in this field.

Most of the biomarkers in HNSCC have been explored retrospectively, often using baseline archival tumor samples at a single time point which may not reflect the impact of spatial and temporal intratumoral heterogeneity. Also, standalone evaluation of potential biomarkers without considering interactions with other factors is likely oversimplifying the complexity of immune response. The microbiota is a dynamic and complex ecosystem that interrelates the immune system and the TME, thus, potentially representing an ideal biomarker that reflects the interactions between these biological entities in totality. Both oral and intestinal microbiota may be important regulators of local and systemic immune responses induced by environmental factors, shaping the TME and ultimately modulating the efficacy of cancer therapies. Considering the emerging immunomodulatory effects of the microbiota, the study of its role as a predictive immune biomarker in HNSCC is of special interest and should be integrated into prospective clinical trials.

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Original Research

Impact of cisplatin dose and smoking pack-years in human papillomavirus–positive oropharyngeal squamous cell carcinoma treated with chemoradiotherapy

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Outcome

Abstract ***Background:*** To evaluate the impact of cisplatin cumulative dose (CDDP-D) and smoking pack-years (PYs) on cause-specific survival (CSS) and overall survival (OS) in human papillomavirus–positive (HPV+) oropharyngeal carcinoma (OPSCC) using the eighth edition tumour-node-metastasis (TNM) staging classification (TNM8).
Patients and methods: We reviewed patients with HPV+ OPSCC treated with high-dose CDDP and intensity-modulated radiotherapy between 2005 and 2015 at Princess Margaret Cancer Centre. CSS and OS were compared according to CDDP-D <200/=200/>200 mg/m^2 stratified by TNM8.
Results: A total of 482 consecutive patients were evaluated (stage I/II/III: N = 189/174/119; CDDP-D <200/=200/>200 mg/m^2: N = 112/220/150). Median follow-up duration was 5.1 years (range: 0.6–12.8). Five-year CSS and OS differed by stages I/II/III: 96%/85%/88% (p=0.005) and 93%/84%/78% (p = 0.001), respectively. Five-year CSS by CDDP-D <200/=200/>200 mg/m^2 was similar in stage I (98%/95%/95%, p = 0.74) and stage II (88%/84%/

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84%, $p = 0.86$) but different in stage III (76%/98%/84%, $p = 0.02$). Five-year OS by CDDP-D <200/=200/>200 mg/m^2 did not differ significantly among stages. In the multivariable analysis, CDDP-D <200 mg/m^2 did not influence CSS in the whole cohort versus = 200/>200 mg/m^2 ($p=0.53/0.79$, respectively) but was associated with reduced CSS in stage III subgroup versus =200 mg/m^2 (=200 mg/m^2 versus < 200 mg/m^2 hazard ratio [HR] = 0.08; 95% confidence interval [CI]: 0.01–0.67; $p = 0.02$). Higher smoking PYs had no effect on CSS ($p = 0.34$) but reduced OS in the whole cohort (HR = 1.14 [95% CI: 1.02–1.27], $p=0.01$).
Conclusion: CDDP-D correlated with neither survival nor disease-specific outcomes in this large and homogeneous HPV+ cohort, although reduced CSS was observed in stageIII HPV+ OPSCC receiving CDDP-D <200 mg/m^2. Smoking PYs were negatively associated with OS but not with CSS.

1. Introduction

Human papillomavirus–positive (HPV+) oropharyngeal squamous cell carcinomas (OPSCCs) present a unique biological behaviour characterised by increased radiosensitivity and improved overall survival (OS) when compared with HPV-negative (HPV−) head and neck squamous cell carcinomas (HNSCCs) [1,2]. This disparity in prognosis was not captured in the 7th edition of American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) tumour-node-metastasis (TNM) staging system and led the head and neck community to develop new staging criteria for HPV+ OPSCC [3]. The 8th edition TNM (TNM8) provides a more accurate prognostic classification that could lead to a better patient selection and tailored therapeutic approach in the era of de-escalation clinical trials for HPV+ OPSCC [4–7].

Concurrent chemoradiotherapy (CRT) with high-dose cisplatin (CDDP) remains the standard of care for locoregionally advanced OPSCC (LA-OPSCC) regardless of HPV status [8]. The modest survival benefit of CRT versus radiation is accompanied by significant acute and long-term toxicity that often compromises treatment tolerance, with a considerable number of patients unable to receive all 3 cycles of CDDP during standard fractionation radiotherapy [9–11]. A pooled analysis of more than 600 patients with LA-HNSCC treated at the Princess Margaret Cancer Centre in Canada and Istituto Nazionale Tumori in Italy showed that CDDP cumulative dose (CDDP-D) <200 mg/m^2 was associated with reduced OS in HPV− but not HPV+ disease with only a trend observed in patients with HPV+ OPSCC within the T4/N3 subgroup [12]. In addition, smoking pack-years (PYs) was shown to reduce OS in the HPV+ patients, consistent with other studies [13–15]. However, the end-point of OS can be confounded by the comorbid effects of long-term smoking and, as such, cause-specific survival (CSS) may be more appropriate to differentiate deaths due to cancer from tobacco-associated comorbidities and mortalities. Likely for the aforementioned reasons, smoking PYs was not included in the TNM8 classification of HPV+ OPSCC, and continued evaluation of the impact of smoking in this patient population is needed to understand its prognostic relevance.

In this study, a retrospective analysis of a large and homogeneous cohort of patients with HPV+ LA-OPSCC originally staged by TNM7 and treated with concurrent CDDP-based CRT was conducted to evaluate the impact of CDDP-D and smoking PYs on OS and CSS across TNM8 stages. In addition, the effect of smoking exposure on the risk of local, regional and distant recurrence as well as cause of death was examined.

2. Patient and methods

2.1. Study population and design

Newly diagnosed HPV+ OPSCC and carcinoma of unknown primary (CUP) with HPV+ cervical lymphadenopathy treated with concurrent high-dose CDDP-based CRT between 2005 and 2015 were identified from our in-house Anthology of Outcome Database [16]. Patient receiving other chemotherapy agents or weekly schedule were excluded. A retrospective chart review of CDDP-D and toxicity was conducted by a single rater, with 50 patients independently audited by a second rater. Concordance was 96%. Discordance was settled by consensus. HPV status was determined by p16 staining and classified as positive if there is nuclear and cytoplasmic staining in ≥70% tumour cells. In situ hybridisation to confirm the presence of high-risk HPV DNA was performed in equivocal cases. All patients were initially staged and treated according to TNM7 and re-classified by TNM8 for this study. This study was approved by the institutional research ethics board and included 283 patients from our previously reported analysis [12].

Table 1
Cohort characteristics and outcomes stratified by CDDP-D.

Variables	All patients *(N=482)*	CDDP-D (mg/m^2)			*p* value
		<200 (N=112)	*=200 (N=220)*	*>200 (N=150)*	
Median age (range)	57.1 (31.3, 74.4)	59.6 (40.8, 71.5)	57.5 (34.6, 74.4)	56.7 (31.3, 73.5)	**<0.001**
Gender (%)					0.074
Male	408 (85)	87 (78)	190 (86)	131 (87)	
Female	74 (15)	25 (22)	30 (14)	19 (13)	
ECOG (%)					0.51
0–1	466 (97)	109 (97)	215 (98)	142 (95)	
>/ = 2	16 (3)	3 (3)	5 (2)	8 (5)	
Smoking status (%)					0.4
Current	122 (25)	31 (28)	47 (21)	44 (29)	
Former	194 (40)	42 (38)	97 (44)	55 (37)	
Non-smokers	165 (34)	39 (35)	75 (34)	51 (34)	
Unknown	1	0	1	0	
Smoking pack-years (%)					
Median (range)	10 (0, 100)	8 (0, 80)	9 (0, 100)	10 (0, 90)	0.75
≤10 versus > 10	255 (53) versus 226 (47)	60 (54) versus 52 (46)	116 (53) versus 103 (47)	79 (53) versus 71 (47)	0.99
≤20 versus > 20	333 (69) versus 148 (31)	74 (66) versus 38 (34)	155 (71) versus 64 (29)	104 (69) versus 46 (31)	0.67
≤30 versus > 30	393 (82) versus 88 (18)	82 (73) versus 88 (27)	185 (84) versus 34 (16)	126 (84) versus 24 (16)	**0.035**
Median LDH (range)	299 (111, 502)	196 (125, 502)	204 (111, 429)	194 (128, 419)	0.42
Primary (%)					0.75
Tonsil	255 (53)	53 (47)	121 (55)	81 (54)	
Base of the tongue	189 (39)	50 (45)	83 (38)	56 (37)	
Other	9 (2)	1 (1)	5 (2)	3 (2)	
CUP	29 (6)	8 (7)	11 (5)	10 (7)	
T 8th Ed. (%)					0.91
T0-2	273 (57)	62 (55)	124 (56)	87 (58)	
T3	128 (27)	28 (25)	62 (28)	38 (25)	
T4	81 (16)	22 (20)	33 (15)	25 (17)	
N 8th Ed. (%)					0.33
N0	11 (2)	5 (4)	4 (2)	2 (1)	
N1	287 (60)	70 (62)	132 (60)	85 (57)	
N2	137 (28)	29 (26)	65 (30)	43 (29)	
N3	47 (10)	8 (7)	19 (9)	20 (13)	
TNM8 (%)					0.6
I	189 (39)	45 (40)	84 (38)	60 (40)	
II	174 (36)	39 (35)	87 (40)	48 (32)	
III	119 (25)	28 (25)	49 (22)	42 (28)	
TNM7 (%)					0.25
III	18 (4)	7 (6)	7 (3)	4 (3)	
IVA	407 (84)	92 (82)	192 (87)	123 (82)	
IVB	57 (12)	13 (12)	21 (10)	23 (15)	
RT completion-70Gy (%)					0.19
No	2 (0)	0 (0)	2 (<1)	0 (0)	
Yes	480 (100)	112 (100)	218 (99)	150 (100)	
RT break (%)					0.11
No	400 (83)	89 (79)	180 (82)	131 (77)	
Yes	82 (17)	23 (21)	40 (18)	19 (13)	
Median GTV cc (range)	21.9 (1.1, 219)	22.9 (2.8, 153)	23.9 (1.2, 151)	20.2 (1.1, 219)	0.7
CDDP-D					**<0.001**
Median (range)	200 (80, 300)	175 (80, 190)	200 (200, 200)	280 (225, 300)	
Median follow-up (range)	5.1 (0.67, 12.8)	5.5 (2.3, 11.1)	4.6 (0.6, 12.1)	5.4 (0.7, 12.8)	**0.01**
5-year OS (95% CI)	86% (82–89)	82% (75–91)	88% (83–93)	86% (80–92)	0.31
5-year CSS (95% CI)	90% (86–92)	89% (81–94)	91% (86–94)	88% (82–93)	0.66
5-year DFS (95% CI)	83% (79–86)	77% (69–85)	85% (80–90)	84% (78–90)	0.14
5-year LRC (95% CI)	96% (94–97)	97% (93–99)	97% (92–98)	93% (87–97)	0.77
5-year DC (95% CI)	89% (85–91)	87% (78–92)	89% (84–93)	90% (83–94)	0.88
5-year late toxicity (95% CI)	21% (17–25)	18% (12–27)	24% (18–31)	20% (14–28)	0.70
2-year PEG dependency (95% CI)	5% (3–7)	6% (3–13)	5% (3–9)	4% (2–9)	0.32
Cause of death (%)					0.22
Index cancer	47 (65)	12 (52)	18 (67)	17 (77)	
Other cancer	10 (14)	4 (17)	3 (11)	3 (14)	
Other cause	15 (21)	7 (31)	6 (22)	2 (9)	

Significant p values (<0.05) are highlighted in bold.
Abbreviations: TNM8 = 8th edition UICC/AJCC TNM staging criteria; TNM7 = 7th edition UICC/AJCC TNM staging criteria; CUP = cancer of unknown primary in the neck; RT = radiotherapy; GTV = gross tumour volume; CDDP-D = cisplatin cumulative dose; OS = overall survival; CSS = cause-specific survival; DFS = disease-free survival; LRC = locoregional control; DC = distant control; PEG = percutaneous endoscopic gastrostomy.

2.2. Treatment and follow-up assessment

All patients were treated with intensity-modulated radiotherapy (IMRT) to a gross tumour dose of 70 Gy in 35 fractions over 7 weeks (2 Gy/fraction). Concurrent three-weekly CDDP (100 mg/m^2) was planned on RT days 1, 22 and 43 according to institutional protocols. Local and regional recurrences were confirmed histologically, while distant metastases were diagnosed by unequivocal clinical/radiologic evidence ± histologic confirmation. Survival status was further linked to the Ontario Population-Based Cancer Registry.

2.3. Statistical analysis

For comparisons of clinical characteristics, Fisher exact test was used for categorical variables and Kruskal-Wallis test for continuous variables. Survival end-points including OS, disease-free survival (DFS), and percutaneous endoscopic gastrostomy (PEG) dependency rates were estimated using Kaplan-Meier methods. CSS (death from index cancer) was estimated using the competing risk method. Locoregional control (LRC), distant control (DC) and actuarial rate of grade 3 and 4 late toxicity according to Radiation Therapy Oncology Group/Eastern Cooperative Oncology Group criteria were calculated by the competing risk method (considering death without an event as a competing risk). Outcome parameters were defined from date of diagnosis to date of death or last follow-up. Late toxicity and PEG dependency were calculated from date of CRT completion to date of death or last follow-up.

Clinical end-points were compared by log-rank test between CDDP-D <200, = 200 and > 200 mg/m^2 and stratified by TNM8 stage I, II and III. Cox proportional hazards regression model was used for OS, and Fine-Gray competing risk regression model was used for CSS. Multivariable analyses (MVAs) were performed to explore potential predictors for OS and CSS including CDDP-D (as >200 versus = 200 versus < 200 mg/m^2, as ≥200 versus < 200 and continuous), age (continuous), smoking PYs (>10 versus ≤ 10, as >20 versus ≤ 20, >30 versus ≤ 30 and continuous per 10) and stage. Power analyses were conducted to evaluate the association between OS and key risk factors for the entire cohort. Based on the power calculation, this study would have 86% power to identify a significant association with two-sided significance level at 0.05 and effect size (HR) of 0.7. We performed exploratory MVA to evaluate the impact of CDDP-D and smoking PYs by stage. All tests were two-sided, and results were considered significant if the pvalue was <0.05.

3. Results

3.1. Clinical characteristics

A total of 482 of 560 consecutive patients diagnosed with LA-OPSCC were eligible for the study (Fig. S1). Clinical characteristics and outcome are summarised in Table 1. Overall, patient characteristics were similar when stratified by CDDP-D. In the entire cohort, the main reasons for CDDP-D reduction/delay were myelotoxicity (38%), weight loss (20%) and ototoxicity (12%). Osteoradionecrosis was the most common late toxicity (6%) (Table S1).

3.2. Outcome stratified by stage

Median follow-up duration was 5.1 years (range: 0.6–12.8). Statistically significant differences in 5-year CSS and OS were observed by stage I, II and III ($p=0.005$ and $p<0.001$, respectively) (Fig. 1A, Table S2, Fig. S2A). While LRC remained similar across stages I, II and III ($p=0.25$), DC was significantly higher in stage I ($p=0.015$). Late toxicity and PEG dependency rate at 2 years significantly increased by stage ($p=0.005$ and $p<0.001$, respectively). Index cancer was the most frequent cause of death in the entire cohort (65%). Fifteen patients (21%) died from other causes, while nearly one-sixth of the deaths were caused by second primary malignancies (14%) (Table S2 and S3).

3.3. Impact of cisplatin dose

Five-year CSS and OS did not differ across patients receiving <200, = 200 and > 200 mg/m^2 ($p=0.66$ and $p=0.315$, respectively) in the entire cohort (Table 2, Fig. 1B, Fig. S2B). Similarly, MVA results adjusted for age, stage and smoking PYs showed that CDDP-D did not affect OS or CSS ($p = 0.35$ and $p=0.59$, respectively).

In univariable analysis, no significant differences were observed in other outcome parameters including DFS, LRC and DC. Late toxicity and PEG dependency rate at 2 years also did not differ by CDDP-D <200, = 200 and > 200 mg/m^2 ($p=0.70$ and $p=0.32$, respectively) (Table 1). Cause of death by CDDP-D was similar, although the proportion of deaths due to index cancer trended higher among patients with CDDP-D >200 mg/m^2 (52% versus 67% versus 77%, $p=0.22$).

In subgroup analysis by stage, 5-year CSS was significantly lower in patients with stage III disease receiving CDDP-D <200 mg/m^2 (76%) than in those receiving = 200 mg/m^2 (98%) and >200 mg/m^2 (84%) (p = 0.022), with a trend towards decreased 5-year OS (65% versus 89% versus 74% for <200 versus = 200

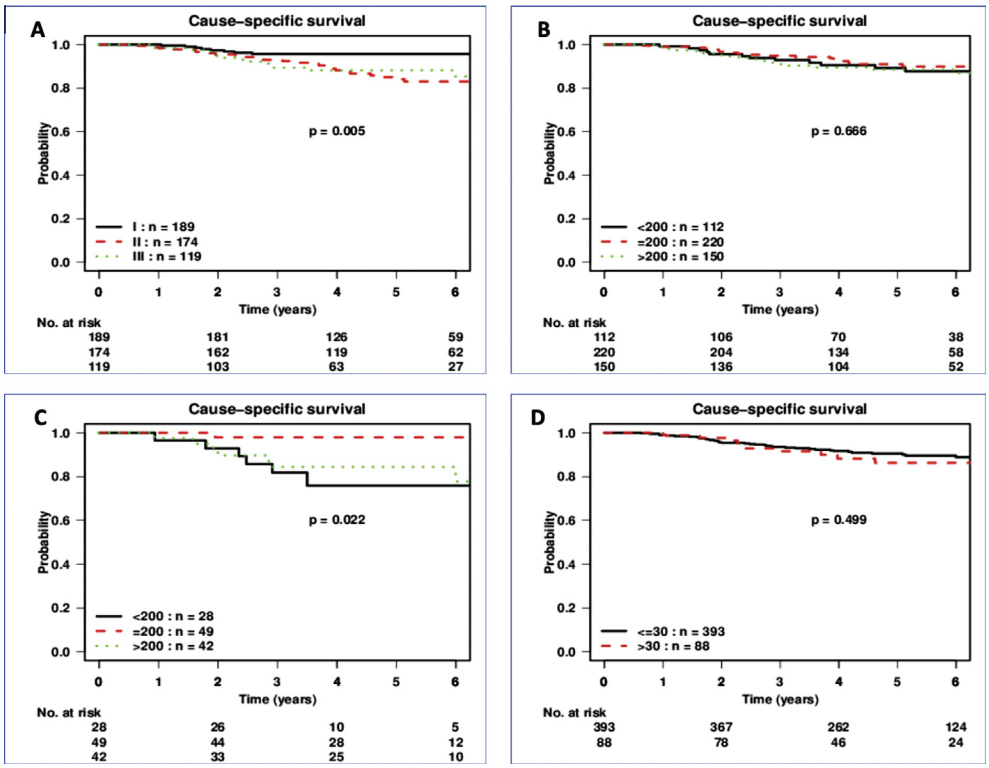

Fig. 1. Kaplan-Meier plots for 5-year CSS for (A) the entire cohort stratified by stage I, II and III; (B) the entire cohort stratified by CDDP-D (>200 versus = 200 versus < 200 mg/m^2); (C) stage III stratified by CDDP-D (>200 versus = 200 versus < 200 mg/m^2) and (D) the entire cohort stratified by smoking PYs ≤30 versus > 30. OS = overall survival; PY = pack-year; CSS = cause-specific survival.

versus > 200 mg/m^2, respectively, p = 0.09) (Table 2, Fig. 1C, Fig. S2C).

3.4. Impact of smoking pack-years

Smoking PYs partitioned at 10, 20 and 30 PYs did not impact 5-year CSS in the entire cohort or by stage (Table 2, Fig. 1D, data on 10 and 20 PYs not shown). No consistent findings were noted in MVA results for CSS when adjusted for age, stage and CDDP-D.

A significantly lower 5-year OS was observed among patients with smoking PYs >30 versus ≤ 30 (75% versus 88%, *p=0.017*) in the entire cohort regardless of stage (Table 2, Fig. S2D). In the MVA for the entire cohort adjusted for age, stage and CDDP-D, smoking PYs (continuous by 10) had a detrimental impact on OS (HR: 1.14 [95% CI: 1.02–1.27] *p=0.01*), and a similar trend was observed when using smoking PYs partitioned at 30 PYs (HR > 30 versus ≤ 30 PY: 1.59 [95% CI: 0.92–2.74], *p=0.09*). No significant correlation was seen between smoking PYs and other outcome parameters including SDS, LRC, DC or late toxicity.

4. Discussion

This single-institution, non-randomly assigned cohort study of patients with HPV+ OPSCC treated with standard-of-care CRT does not show a significant correlation between cumulative CDDP dose and survival- or disease-specific outcomes. Increased smoking pack-years is associated with reduced OS but not CSS.

An association between OS and CDDP-D has been described in few retrospective analyses involving heterogeneous patient population with LA-HNSCC treated with either definitive or postoperative CRT [17,18]. Whether the survival gain with increasing CDDP-D can be attributed to improved LRC and/or DC is unclear [12,18]. Two prospective randomised studies evaluating the role of RT plus cetuximab versus CRT as a de-escalation approach in HPV+ LA-OPSCC revealed significantly higher OS, LRC and DC in the CDDP arm regardless of stage [19,20]. However, the optimal cumulative CDDP-D and the question of whether all patients with HPV+ OPSCC needed CDDP were not addressed. The only phase III prospective study evaluating CDDP dose and schedule in LA-HNSCC indicated the relevance of these parameters in LRC although it is not fully applicable to our present study because it included mainly patients treated in the post-operative adjuvant setting with oral cavity primaries [21].

The overall impact of CDDP-D on treatment outcome and survival in patients with HPV+ LA-OPSCC remains unknown. A non-inferiority prospective comparison of two versus three cycles of CDDP-D in HPV+ disease will unlikely be pursued given the

Table 2
Impact of cisplatin dose and smoking pack-year on OS and CSS in the entire cohort and stratified by stage.

CDDP-D (mg/m^2)		Entire cohort (N = 482)	Stage (TNM8)		
			I (N = 189)	II (N = 174)	III (N = 119)
OS	**5-year OS (95% CI)**				
	<200	82% (75−91)	88% (78−100)	86% (75−98)	65% (47−89)
	=200	88% (83−93)	94% (88−99)	83% (74−92)	89% (81−99)
	>200	86% (80−92)	95% (89−100)	84% (74−96)	74% (61−89)
	p value	*0.31*	*0.13*	*0.81*	*0.09*
	MVA HR (95% CI)				
	=200 versus < 200	0.66 (0.38, 1.16 *p=0.15*)	0.57 (0.18, 1.75 *p=0.32*)	1.20 (0.49, 2.97 *p=0.69*)	0.36 (0.13, 1.03 *p=0.05*)
	>200 versus < 200	0.74 (0.41, 1.35 *p=0.33*)	0.31 (0.07, 1.34 *p=0.12*)	0.92 (0.32, 2.64 *p=0.88*)	0.91 (0.38, 2.18 *p=0.83*)
	≥200 versus < 200	0.70 (0.42, 1.15 *p=0.16*)	0.46 (0.16, 1.31 *p = 0.15*)	1.10 (0.46, 2.58 *p=0.84*)	0.61 (0.27, 1.36 *p=0.22*)
CSS	**5-year CSS (95% CI)**				
	<200	89% (81−94)	98% (84−100)	88% (70−96)	**76% (49−89)**
	=200	91% (86−94)	95% (87−98)	84% (72−90)	98% (85−100)
	>200	88% (82−93)	95% (84−98)	84% (69−92)	84% (67−93)
	p value	*0.66*	*0.74*	*0.86*	***0.02***
	MVA HR (95% CI)				
	=200 versus < 200	0.79 (0.38, 1.63 *p=0.53*)	2.59 (0.25, 26.9 *p=0.42*)	1.39 (0.52, 3.77 *p=0.51*)	**0.08 (0.01, 0.67 *p = 0.02*)**
	>200 versus < 200	1.11 (0.52, 2.34 *p=0.79*)	2.89 (0.23, 35.6 *p=0.41*)	1.03 (0.42, 4.08 *p=0.65*)	0.76 (0.2, 2.23 *p=0.62*)
	≥200 versus < 200	0.92 (0.48, 1.77 *p=0.80*)	2.71 (0.27, 26.98 *p=0.39*)	1.36 (0.53, 3.51 *p=0.52*)	0.38 (0.13, 1.07 *p = 0.066*)
Smoking PYs					
OS	**5-year OS (95% CI)**				
	≤30 versus > 30	**88% (85−92)** versus **75% (65−86)**	94% (90−98) versus 84% (71−100)	85% (80−92) versus 68% (48−97)	82% (74−91) versus 70% (54−89)
	p value	***0.01***	*0.07*	*0.29*	*0.33*
	MVA HR (95% CI)				
	Continuous per 10	**1.14 (1.02, 1.27 *p = 0.01*)**	1.18 (0.96, 1.78 *p=0.12*)	1.20 (0.97, 1.49 *p=0.1*)	1.09 (0.92, 1.29 *p=0.31*)
	≤20 versus >20	1.39 (0.85, 2.26 *p=0.19*)	1.65 (0.61, 4.46 *p=0.33*)	1.41 (0.62, 3.21 *p=0.41*)	1.19 (0.56, 2.56 *p=0.65*)
	≤30 versus > 30	1.59 (0.92, 2.74 *p=0.09*)	2.31 (0.77, 6.9 *p=0.13*)	1.66 (0.32, 2.84 *p=0.32*)	1.26 (0.57−2.79 *p=0.57*)
CSS	**5-year CSS (95% CI)**				
	≤30 versus >30	90% (87−93) versus 86% (75−92)	97% (92−99) versus 90% (71−97)	87% (80−92) versus 68% (30−86)	86% (76−92) versus 94 (75−98)
	p value	*0.49*	*0.11*	*0.14*	*0.20*
	MVA HR (95% CI)				
	Continuous per 10	1.08 (0.92, 1.26 *p=0.34*)	1.27 (0.86, 1.88 *p=0.23*)	**1.27 (1.01, 1.58 *p = 0.03*)**	0.84 (0.66, 1.06 *p=0.14*)
	≤20 versus >20	1.26 (0.67, 2.37 *p=0.47*)	1.39 (0.33, 5.81 *p=0.65*)	1.78 (0.78, 4.05 *p=0.17*)	0.66 (0.23, 1.90 *p=0.45*)
	≤30 versus > 30	1.27 (0.58, 2.78 *p=0.55*)	3.48 (0.79, 15.33 *p=0.09*)	2.25 (0.77, 6.59 *p=0.14*)	0.29 (0.07, 1.19 *p=0.08*)

Abbreviations: MVA = multivariable analysis; TNM8 = 8th edition UICC/AJCC TNM staging criteria; CDDP-D = cisplatin cumulative dose; PYs = pack-years; OS = overall survival; CSS = cause-specific survival; HR = hazard ratio.
Note: MVA for OS was adjusted for age, stage and smoking PYs. MVA for CSS was adjusted for stage and smoking PYs. MVA for TNM8 subgroups includes CDDP-D and smoking PYs.
Note: Significant *p*-values are in bold.

number of patients required; hence, our report represents the largest retrospective cohort specifically interrogating this question.

In our previous retrospective study including more than 600 patients with HNSCC treated with primary CRT, we found no significant correlation between CDDP-D and OS or other disease-control outcomes in the HPV+ subgroup [12]. The current analysis involved a larger, homogeneous cohort of patients with HPV + LA-OPSCC uniformly treated with IMRT which excluded patients treated with weekly CDDP and other chemotherapy agents. CDDP-D (<200 mg/m^2, $=$ 200 mg/m^2 or >200 mg/m^2) had no significant effect on 5-year OS, 5-year CSS or any of the other outcome parameters including DFS, LRC and DC in the entire cohort, but the present study is underpowered for effect sizes of HR < 0.7. In the exploratory subgroup MVA by stage, CDDP-D <200 mg/m^2 was associated with reduced CSS in patients with stage III disease despite no decrease in either LRC and/or DC was observed. Overall, CSS remained poor in stage III subgroup regardless of CDDP-D, mainly because of reduced DC. These results suggest that the therapeutic benefit of standard-of-care CRT might have reached a plateau and support the need for chemo-additive strategies in stage III HPV+ OPSCC such as immunotherapy-based CRT approaches being explored in ongoing clinical trials (*NCT02952586, NCT03040999*). Data on de-intensification are not yet mature to support de-escalation strategies outside of prospective clinical trials.

We additionally analysed the impact of smoking in our cohort as its role as a prognostic biomarker for risk stratification in HPV+ disease remains controversial [1,22]. In retrospective analyses of heterogeneous cohorts of patients with LA-OPSCC treated with different treatment modalities, smoking negatively impacted OS and DC, while other studies failed to show a correlation with CSS and disease-control outcomes in HPV+ patients [13,15,23,24]. In more than 200 patients with TNM8 I to III HPV+ OPSCC treated with RT or CRT, smoking status and smoking PYs partitioned at either >10 or ≥ 20 were strong negative prognostic factors in the MVA for OS and DFS and were significantly correlated with lower LRC and DC, but the effect on CSS was not evaluated [14]. In our study, neither smoking status nor PY (continuous by 10 or partitioned at 10, 20 and 30 PYs) impacted 5-year CSS or any disease-specific outcome parameter (DFS, LRC and DC). However, smoking PY (continuous by 10) was found to be an independent negative prognostic factor in the MVA analysis for OS, and >30 PYs smoking history was significantly associated with lower 5-year OS. The majority of the patients in our cohort had a history of smoking, with a quarter of them being active smokers at the time of diagnosis, similar to previous studies involving HPV+ patients [14,15]. The distribution of the smoking variables was also similar across stage and CDDP-D subgroups, therefore minimising their potential confounding effect. Our data on the differential impact of smoking history on 5-year CSS and OS raise the importance of considering both parameters as efficacy end-points. Smoking has a direct impact on overall health, and comorbidities are associated with decreased survival in HNSCC regardless of treatment intervention and stage [25]. Among patients with HNSCC, smokers are at higher risk of developing secondary malignancies, especially younger patients who more frequently present with HPV+ disease [26,27]. In our study, 14% of the deaths were caused by second malignancies including lung, oesophagus and head and neck, commonly smoking-related cancers. This percentage remained similar across stage and CDDP-D subgroups and might explain the differential impact of smoking on OS and CSS. Smoking affects RT efficacy and toxicity, which may ultimately affect CSS [28,29]. Despite the well-known implications of smoking in carcinogenesis and immunosuppression, the role that tobacco plays in the biology of HPV+ OPSCC has not yet been elucidated and the few retrospective studies comparing the genomic and immune landscapes of HPV+ tumours in smokers versus non-smokers have shown inconsistent results [30–32].

Despite the large, selected and homogeneously treated patients evaluated, we acknowledge the limitations inherent to the retrospective nature of this study. While the oncologic outcomes were recorded prospectively, CDDP-D and toxicity rates were collected retrospectively and potential confounders, including patient compliance, social and economic factors, were unavailable. Although cause of death was prospectively attributed based on death certificate and treating clinician's interpretation, mis-attribution could not be entirely excluded because of the challenges in determining underlying cause of death in a few cases. The study was underpowered to detect differences in specific subsets given the small sample size in some subgroups.

The authors believe that the results of this study are of particular relevance for current practice and may contribute to guiding risk stratification and new treatment strategies within clinical trials. While awaiting prospective data, CRT should remain the standard of care in this patient population, although treatment intensification approaches should be pursued when available for patients with stage III disease. Smoking was correlated with patients' OS but not CSS or disease-control outcomes in HPV+ disease; hence, its role in risk stratification and treatment selection should be investigated in prospective studies.

Conflict of interest statement

A.S. has served the role of a consultant for Merck (compensated), Bristol-Myers Squibb (compensated),

Novartis (compensated) and Oncorus (compensated) and has receivedgrant/research support from (clinical trials) Novartis, Bristol-Myers Squibb, Symphogen AstraZeneca / Medimmune, Merck, Bayer, Surface Oncology, Northern Biologics, Janssen Oncology / Johnson & Johnson, Roche and Array Biopharma. L.L.S. has served the role of a consultant for Merck (compensated), Pfizer (compensated), Celgene (compensated), AstraZeneca/Medimmune (compensated), Morphosys (compensated), Roche (compensated), GeneSeeq (compensated), Loxo (compensated), Oncorus (compensated) and Symphogen (compensated); has received grant/research support from (clinical trials) Novartis, Bristol-Myers Squibb, Pfizer, Boerhinger-Ingelheim, Regeneron, GlaxoSmithKline, Roche / Genentech, Karyopharm, AstraZeneca / Medimmune, Merck, Celgene, Astellas, Bayer, Abbvie, Amgen, Symphogen and Intensity Therapeutics; and is a stockholder in Agios (spouse). All remaining authors have declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.06.019.

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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

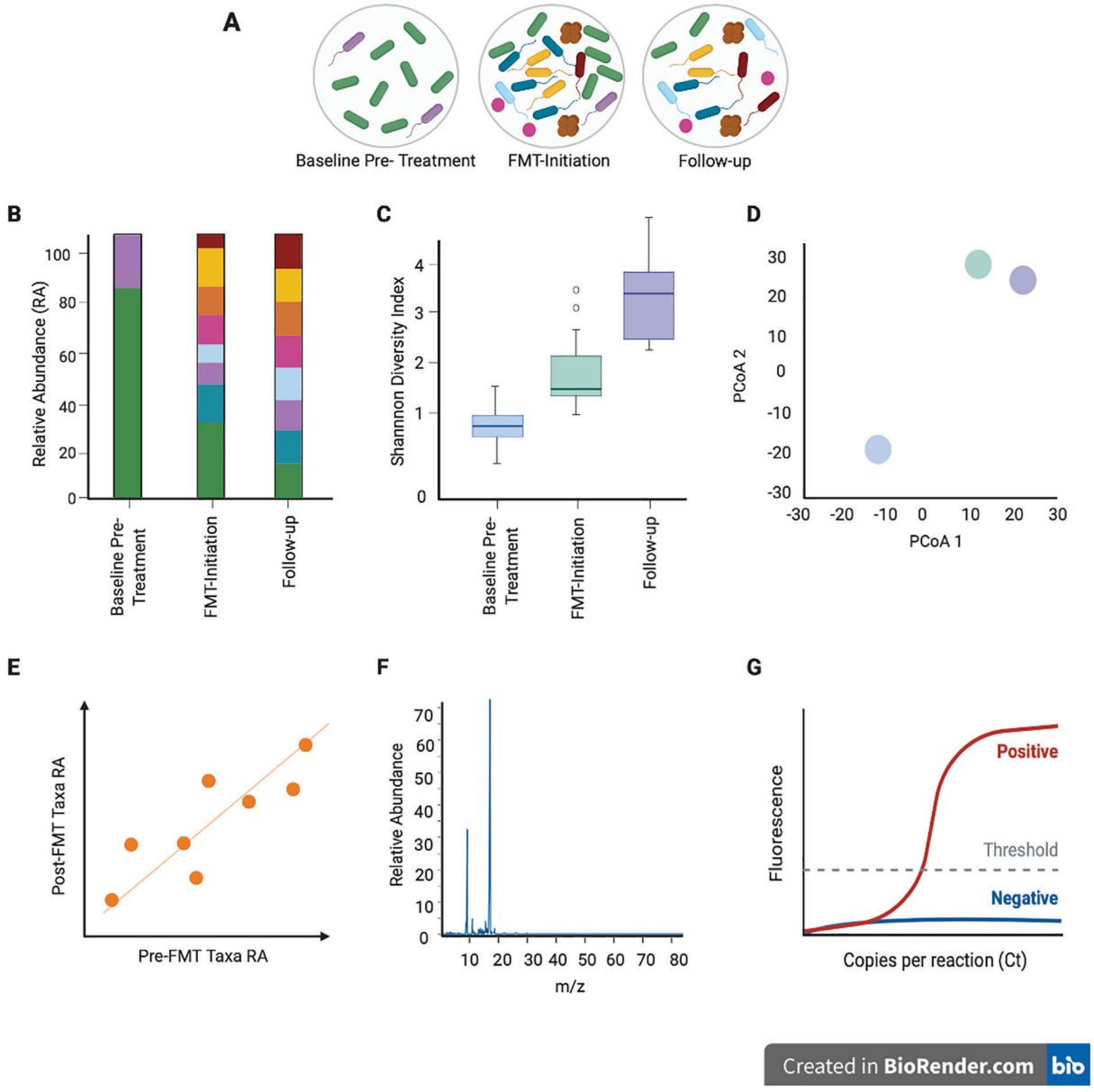

Fig. 1. Measuring microbiome manipulations. A) Hypothetical representation of microbial diversity found in fecal samples at baseline, post-FMT, and at follow-up. B) Relative abundance (RA) of different microorganisms present in panel A. C) Shannon alpha-Diversity index (SDI) (within-sample) showing the 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

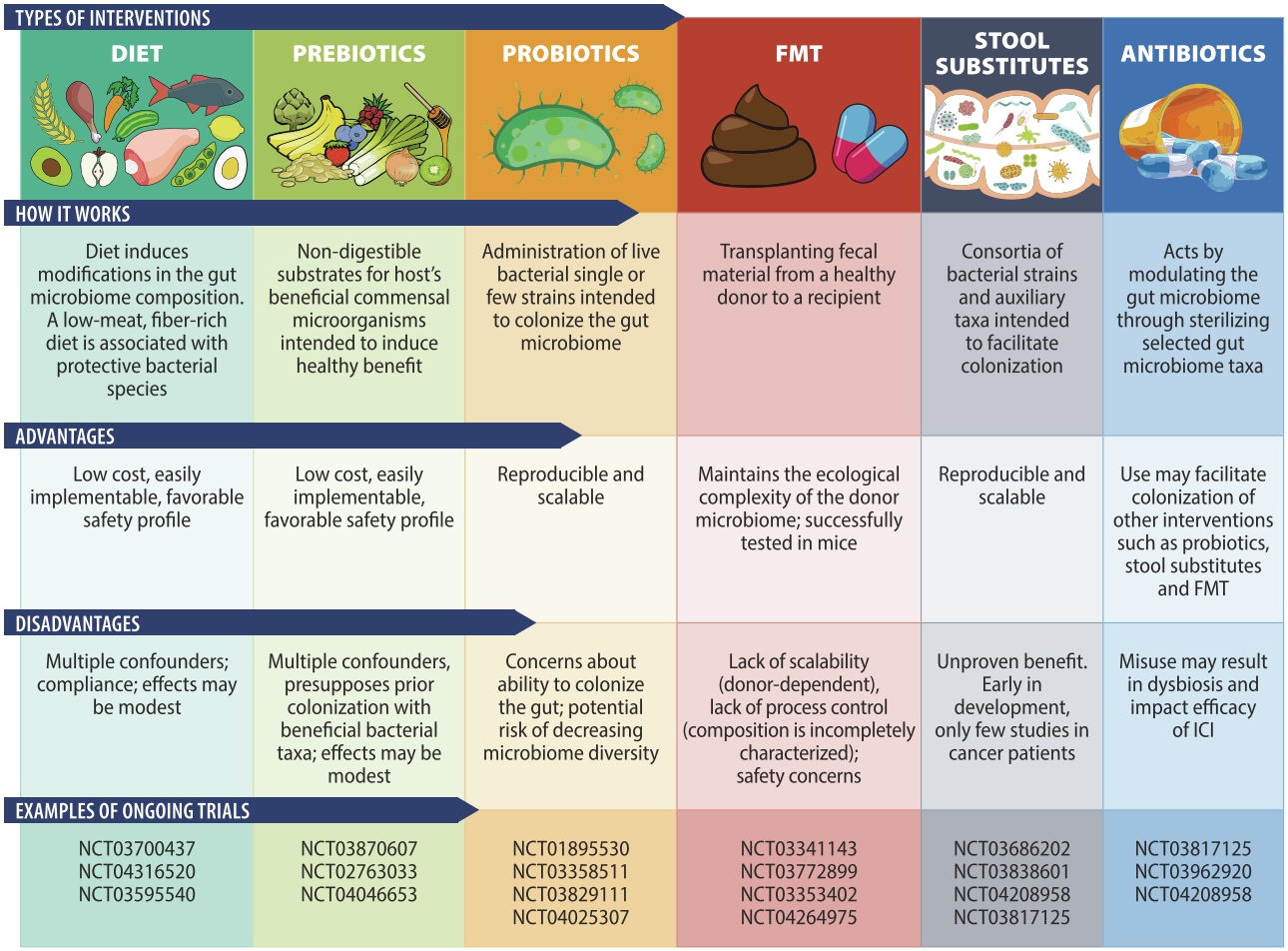

Fig. 2. Summary of the modalities of intervention for modifying the gut microbiome.

[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 meals 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 patients 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

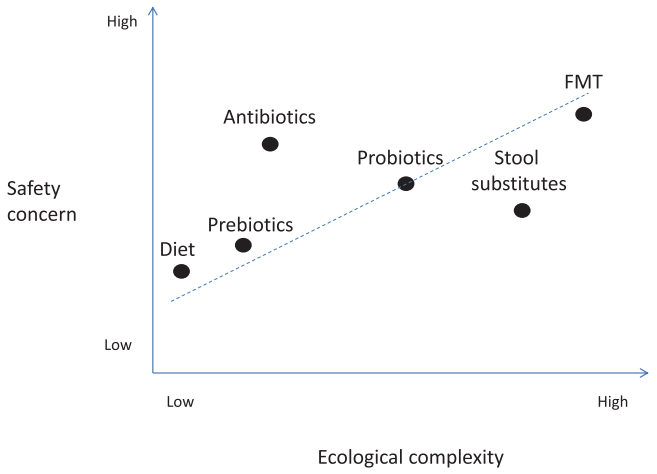

Fig. 3. Potential safety concerns and ecological complexity of microbiome modifying interventions. Higher complexity interventions, such as FMT, have the greatest potential safety issues.

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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Review

Tumor-Associated Microbiome: Where Do We Stand?

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Abstract: The study of the human microbiome in oncology is a growing and rapidly evolving field. In the past few years, there has been an exponential increase in the number of studies investigating associations of microbiome and cancer, from oncogenesis and cancer progression to resistance or sensitivity to specific anticancer therapies. The gut microbiome is now known to play a significant role in antitumor immune responses and in predicting the efficacy of immune-checkpoint inhibitors in cancer patients. Beyond the gut, the tumor-associated microbiome—microbe communities located either in the tumor or within its body compartment—seems to interact with the local microenvironment and the tumor immune contexture, ultimately impacting cancer progression and treatment outcome. However, pre-clinical research focusing on causality and mechanistic pathways as well as proof-of-concept studies are still needed to fully understand the potential clinical utility of microbiome in cancer patients. Moreover, there is a need for the standardization of methodology and the implementation of quality control across microbiome studies to allow for a better interpretation and greater comparability of the results reported between them. This review summarizes the accumulating evidence in the field and discusses the current and upcoming challenges of microbiome studies.

Keywords: tumor microbiome; gut microbiome; dysbiosis; cancer; carcinogenesis; metagenomics

1. Introduction: Microbiology Meets Oncology

This is the "decade of microbiome", reported Forbes' last publication of 2019. While the existing link between microbiome and health in the human host has been known for years, it was not until recently that the influence of the microbiome reached several medical disciplines, including oncology, going from unknown to mainstream.

The human microbiome is defined as the collective genomes and by-products of all the microorganisms inhabiting the human body, including bacteria, viruses, fungi, pro-

tozoa, and archaea [1,2]. These microbial communities are distributed in multiple compartments throughout the body (e.g., skin, oro-gastrointestinal, urogenital tracts) and vary in type and number depending on each compartment, although they all seem to share core functions such as glycolysis, ATP synthesis, and activation of translational machinery [1,2]. The microbiome has a symbiotic and dynamic relationship with the human host, with some microorganisms being key players in several physiological functions that mainly involve regulation of metabolic processes and immune system responses [2]. Although the precise underlying mechanisms are not completely understood, changes in the microbiome composition of a specific body compartment caused by either host intrinsic or external factors (e.g., genetics, infections, diet, or antibiotics) can alter the local homeostasis and induce chronic inflammation, damaging tissues and dysregulating local and systemic immune responses, ultimately leading to disease [3]. These pathology-associated alterations are also known as dysbiosis, and they have been linked to several disorders, including cancer [4].

The association between the microbiome and cancer is not new. Up to 20% of cancers are actually related to infections [5], and several pathogenic bacteria and viruses contribute to the etiopathogenesis of specific tumor types [6–8]. However, beyond these well-established agent-tumor causality associations, overall quantitative and/or qualitative shifts in the microbiome composition of a specific compartment may also trigger cancer initiation, development, and progression. The International Cancer Microbiome Consortium postulates that the microbiome is one apex of a carcinogenesis-leading tripartite, jointly with (epi)genetics and environment [9]. This idea is supported by the Ecological Koch's postulate that sustains that a dysbiosis resulting from (epi)genomics, environment, and microbiome leads to a single disease [10].

In addition to its role in tumorigenesis and cancer progression, the microbiome has also emerged as a new potential biomarker in cancer diagnosis, risk stratification, and prognosis. Microbial signatures detected in cell-free DNA from human fluids have been linked to specific tumor types and could be used for diagnostic purposes [11]. Other studies have shown a correlation between tumor-associated bacteria and survival or response to anticancer therapies [12,13]. Recently, accumulating evidence has implicated the gut microbiome in the modulation of host anticancer immune responses and the efficacy of immune-checkpoint inhibitors across many tumor types [14–17]. These findings have led to preclinical and clinical investigations on how to manipulate the microbiome to use it as a therapeutic tool to boost the efficacy of anticancer therapies through different strategies, from dietary interventions and probiotic/antibiotic therapies to fecal microbial transplantation [3,18] (NCT04264975, NCT01895530, NCT03817125).

2. Tumor-Associated Microbiome

Bacteria, viruses, and other micro-organisms located in different body compartments have been correlated with increased susceptibility of developing different cancers [19–22]. Cancer patients seem to harbor a specific microbiome composition in the tumor niche and also within the tumor's body compartment, which differs from healthy controls [23–26]. These specific changes in the microbial communities intratumor or nearby observed in cancer patients are what we define as tumor-associated microbiome. Whether this tumor-associated microbiome is involved in carcinogenesis or if it is merely a by-stander effect due to the tumor microenvironment is yet to be fully elucidated. It is hypothesized that a dysbiosis in a specific compartment or tissue could start an oncogenic process through (1) the induction of chronic inflammation, (2) the inhibition of cellular apoptosis, (3) the production and release of carcinogenic substances, or (4) the modulation of local anti-tumor immunity and tumor microenvironment [27]. For instance, changes in the relative abundance (RA) of a given group of bacteria has been shown to directly cause DNA damage leading to genetic dysregulation and initiation of tumorigenesis [28,29]. A recent elegant study conducted by Nejman et al. revealed that intra-tumor microbiome composition is diverse and cancer type-specific [23]. Interest-

ingly, bacteria found in tumor tissue were biologically active and mainly located in the cytoplasm of both tumor and immune cells, suggesting an implication in both oncogenesis and antitumor immunity.

In this section, we revise the evidence available on the tumor-associated microbiome by cancer type and its potential clinical use as a diagnostic, prognostic, or predictive biomarker (Table 1).

Table 1. Tumor-associated microbiome.

Disease Site	Tumor Type	Sample Type	Tumor-Associated Taxa	Potential Clinical Utility Based on Recent Evidence
Head and Neck and Upper Gastrointestinal Tumors	Head and neck SCC	Saliva tumor tissue	*Kingella* and *Corynebacterium* [20,30]	Cancer prevention
			Porphyromonas gingivalis [31]	Diagnostic
			Fusobacterium nucleatum [32,33]	Prognostic
	Esophageal ADC	Tumor tissue	*Campylobacter* species [34–36]	Diagnostic
	Esophageal SCC	Tumor tissue	*Fusobacterium nucleatum* [13,33]	Prognostic
	Gastric carcinoma	Tumor tissue	*Helicobacter pylori* [6]	Diagnostic
Hepatocarcinoma and Pancreatic Cancer	Ductal ADC	Tumor tissue	VHB, VHC [5]	Screening Diagnostic
		Normal tissue	*H. pylori*, *P gingivalis*, *Fusobacterium* sp., *Aggregatibacter* sp., *Prevotella* sp., or *Capnocytophaga* sp. [37]	Diagnostic
	Pancreatic ductal ADC	Tumor tissue	*Pseudoxanthomonas* sp., *Streptomyces* sp., *Saccharopolyspora* sp., *Bacillus clausii, Proteobacteri* sp. [26]	Prognostic
			Gammaproteobacteria [38]	Predictive
Colorectal Cancer	Colorectal ADC	Tumor tissue Stool Saliva	*Fusobacterium nucleatum* [39–43]	Diagnostic Prognostic Predictive Therapeutic
		Tumor tissue	Enterotoxigenic *Bacteroides fragilis* [44]	Cancer Prevention
			Escherichia coli (pk+) [45]	
		Stool	*Peptostreptococcus stomatis, Parvimonas, Porphyromonas* [39,40] *Ascomycota, Basidiomycota, Orthobunyavirus* [46–48]	Diagnostic Screening tool
Genitourinary tumors	Urothelial carcinoma	Urine	*Fusobacterium*, Firmicute [49,50]	Diagnostic
	Renal cell carcinoma	Tumor tissue	*Chloroplast*, *Streptophyta* [51]	Diagnostic

	Prostate SDC	Tumor tissue	*Akkermansia muciniphila* [52]	Predictive of response
			Listeria monocytogenes [53]	Prognostic
	Endometrial cancer	Tumor tissue	*Porphyromonas* sp., *Atopobium vaginae* [54]	Diagnostic
Lung cancer	Lung ADC and SCC	Normal site	*Chlamydia pneumonia, Mycobacterium tuberculosi* [55]	Cancer prevention
		Saliva	*Veillonella, Capnocytophaga, Selenomonas Megasphaera, Neisseria* [56]	Diagnostic
		Tumor tissue	Family Lachnospiraceae, genera Faecalibacterium and Ruminococcus [57]	Prognostic
		Faeces	*Akkermansia muciniphila* [14]	Predictive of response
Breast cancer	Triple-positive ductal ADC (HR/HER-2+)	Tumor tissue	*Bordetella*, *Campylobacter*, *Chlamydia*, *Chlamydophila*, *Legionella*, *Pasteurella* [58]	Diagnostic
	Triple-negative ductal ADC (HR/HER-2 -)	Tumor tissue	*Aerococcus*, *Arcobacter*, *Geobacillus*, *Orientia*, *Rothia* [58]	Diagnostic
HPV-related cancers	Oropharyngeal SCC	Saliva	*Lactobacillus*-enriched [32]	Diagnostic
	Cervical SCC	Tumor tissue	HPV16 [5]	Prognostic
		Vaginal fluid	*Lactobacillus*, *Gardnerella*, *Atopobium*, *Fusobacterium, Sneathia* [59]	Diagnostic Prognostic
EBV-related cancers	Nasopharyngeal carcinoma	Tumor tissue	EBV [60]	Diagnostic Prognostic
		Gut	Functional metabolic signature [61]	Prognostic

Abbreviations: SCC = squamous cell carcinoma; ADC = adenocarcinoma; HR = hormonal receptors; HPV = human papillomavirus; EBV = Epstein–Barr virus.

2.1. Cancers of the Upper Aerodigestive Tract: Head and Neck and Esophageal Tumors

Head and neck, esophageal, and gastric cancers arise from the epithelium and mucosa of the oro-gastrointestinal tracts and upper airway. These compartments are constantly exposed to external aggressions such as smoking, alcohol consumption, or infections, which can alter their microbiome composition [62,63]. Some viral infections such as Epstein–Barr virus (EBV) and human papillomavirus (HPV) are well-established etiopathological agents of nasopharyngeal (NPC) and oropharyngeal carcinomas (OPC), respectively [60,64], while the bacterial species *Helicobacter pylori* (*H. pylori*) is causally associated with the incidence of gastric adenocarcinomas and mucosa-associated lymphoid tissue lymphoma [6,65].

However, beyond these specific pathogen–tumor type causality relationships, which will be further discussed, oro-gastrointestinal dysbiosis has been correlated with increased risk of head and neck (HNC), esophageal (EC), and gastric cancers (GC) [66–69]. Several retrospective case–control studies have found differential microbiome composition in the saliva, mucosal, and tumor tissues of patients with these tumor types when compared to healthy individuals, suggesting an implication in tumor initiation and development [25,70–72]. Other commensal bacteria have been shown to be protective of

cancer development and could be used for cancer prevention purposes—both *Kingella* and *Corynebacterium* species, which are functionally implicated in the biodegradation and/or metabolization of carcinogens from tobacco and/or alcohol (e.g., Acetaldehyde), have been linked with decreased risk of head and neck squamous cell carcinomas (HNSCC) among smokers/alcohol consumers in a nested case–control study within a prospective cohort [20,30].

Oropharyngeal and esophagogastric compartments share similar commensal microorganisms as well as microbiome pathogenic alterations [73]. For instance, increased RA of oral *Porphyromonas gingivalis*, a bacterium associated with periodontal disease, has been suggested to facilitate the development of oral carcinomas through the activation of immune evasion mechanisms and oncogenic pathways, but it also has been correlated with cancer cell differentiation and metastasis in patients with esophageal squamous cell carcinoma (SCC) [31,74]. *Fusobacterium nucleatum (F. nucleatum)* is also found in both head and neck and esophageal SCC and is associated with advanced tumor stages and a more aggressive tumor behavior in both patient populations [32,33,75]. In contrast, different microbiome composition has been described within the same compartment in association with a specific tumor histology, indicating that intercompartmental dysbiosis might lead to different tumor types or vice versa; whether these findings are cause or consequence is yet unclear. *Campylobacter* species are found increased in the esophageal mucosa of patients with gastroesophageal reflux disease (GERD) and Barrett's esophagus, and seem to be implicated in the development of esophageal adenocarcinomas but not SCC through the activation of immune pathways linked to toll-like receptors [34–36]. In the case of EC and GC, infections by other microorganisms such as fungi have also been implicated in carcinogenesis through mucosal injury and dysregulation of the local immune system and oncogenic pathways [76,77].

Fewer studies are available on the potential impact of tumor-associated microbiome on outcome and response to therapy in patients with cancer of the upper aerodigestive tract. In HNC, there is no evidence of oral/tumor-associated microbiome as a biomarker of response to standard therapies such as radiotherapy, chemotherapy, or immunotherapy, although studies are underway (NCT03410615). To date, the only study that evaluated the oral microbiome in a subgroup of patients with recurrent/metastatic HNSCC treated with antiPD-1 agent nivolumab within the CheckMate-141 clinical trial failed to show any correlation with treatment response [78]. However, the small number of patients and the low percentage of responses might have influenced these results. In terms of toxicity, two studies have shown a correlation between oral dysbiosis and increased radiation-induced mucositis in patients with HNC [79,80]. In patients with esophageal SCC, increased RA of intratumoral *F. nucleatum* has been associated with poor response to neoadjuvant chemoradiation and higher risk of recurrence [13].

2.2. Hepatocarcinoma, Pancreas, and Biliary Tract Cancers

Cancers from the hepato-biliary system are under the influence of the microbiomes belonging to each of the organs involved but also of the gut microbiome via blood flow through the portal vein [37]. The relationships between gut microbiome, biliary acids, and liver diseases, including hepatic steatosis, non-alcoholic liver disease, non-alcoholic steatohepatitis, cirrhosis, biliary tract cancers, and hepatocellular carcinoma (HCC), have been reviewed extensively [81,82]. A recent pre-clinical study by Zhang et al. evaluated the gut microbiome in 127 mouse models for primary sclerosis cholangitis, colitis, and cholangiocarcinoma [83]. They were able to show that Gram-negative commensal bacteria from the gut control the accumulation of hepatic myeloid-derived immunosuppressive cells (MDSCs) through a TLR4/CXCL1/CXCR2129-dependent mechanism and thus contribute to an immune-suppressive microenvironment in the liver [83].

Among viruses, hepatitis B (VHB) and C (VHC) infections are well-established risk factors not only for liver cancer but also for pancreatic ductal adenocarcinoma (PDAC). Alcohol-induced tumors (including HCC and PDAC) were observed to have distinct

microbiome composition from virally induced tumors, suggesting that liver microbiome may differ in response to different etiological factors [84]**.** Beyond viruses, certain pathogenic bacteria such as *H. pylori* and oral periopathogens such as *P gingivalis*, *Fusobacterium* sp., *Aggregatibacter* sp., *Prevotella* sp., or *Capnocytophaga* sp. seem to play a role in the development of PDAC via induction of chronic inflammation, antiapoptotic changes, cell survival, and cell invasion [37]. In this regard, a study by Pushalkar et al. detected specific gut and tumor microbiome in murine models of PDAC, suggesting a potential bacterial translocation from the intestinal tract into the peritumoral milieu [85]. Interestingly, PDAC-associated microbiome as well as gut microbiome were involved in immune-suppression in pancreatic tissue, a characteristic often observed in PDAC. Together, these data suggest that gut and/or tumor microbiome represent a potential therapeutic target to modulate disease progression in PDAC.

The PDAC-associated microbiome appears to also have a prognostic role, although its correlation with the incidence of this disease has not been evaluated sufficiently. Riquelme et al. evaluated the intratumor microbiome composition of PDAC patients according to short-term survival (STS) and long-term survival (LTS), identifying a specific intra-tumoral microbiome signature (*Pseudoxanthomonas–Streptomyces–Saccharopolyspora-Bacillus clausii*) that was predictive of long-term survivorship in both discovery and validation cohorts [26]. Chakladar et al. profiled the intra-tumor pancreatic microbiome through large-scale sequencing data from The Cancer Genome Atlas (TCGA) (187 pancreatic cancer samples). The authors found that the increased prevalence and poorer prognosis of PDAC in males and smokers were linked to the presence of potentially cancer-promoting or immune-inhibiting microbes (most of them belonged to Proteobacteria phylum) [86]. Another study showed that intra-tumor Gammaproteobacteria in PDAC modulates tumor sensitivity to gemcitabine, one of the few active and standard of care chemotherapy drugs used in PDAC [38].

In HCC, a small study evaluated the changes in gut microbiome after antiPD-1 therapy in eight patients with Barcelona Clinic Liver Cancer (BCLC) Stage C disease. Differences in microbiome diversity and composition were observed between responders and non-responders, thus suggesting that gut microbiome dynamics might be predictive of response to these agents in patients with HCC [87].

2.3. Colorectal Cancer

The microbiome in colorectal cancer (CRC) is one of the most studied across malignancies, but its role in the development of this tumor is still a matter of debate. The "Driver-Passenger CRC model" defines as drivers those bacteria with pro-carcinogenic features that are found in pre-malignant lesions or in early CRC, while the term "passengers" refers to bacteria that act as tumor promoters or suppressors in later stages of disease [88]. Among drivers, enterotoxigenic *Bacteroides fragilis* drives tumor growth through different mechanisms encompassed in the so-called alpha-bugs hypothesis, such as DNA damage, induction of cell proliferation, and induction of T helper 17 inflammation [44]. *Escherichia coli*, which is a producer of toxin colibactin *(pk+)*, has also raised interest as a driver, since it may cause toxin-induced DNA damage, promoting a specific CRC mutational profile based on insertions and deletions [45]. *F. nucleatum* is the paradigmatic passenger bacterium because it is rarely detected in adenoma, but it may have a relevant role at latter stages of carcinogenesis [89]. Preclinical studies have shown that this species is capable of activating oncogenic pathways such as MAPK and Wnt [19,90], and to impair antitumor immune response through the activation of NF-κB signature and the interaction with immune-checkpoints [91–93]. Of note, its presence has been also found in synchronous or metachronous liver metastases from CRC primary tumors harboring this bacterium [94], suggesting that *F. nucleatum* could disseminate to other organs/locations via systemic circulation, such as cancer cells. Moreover, *F. nucleatum* is more abundant in right-sided tumors [95] and those with mismatch repair deficiency,

indicating a potential relationship with the mutator phenotype pathway of CRC carcinogenesis [39,40].

Beyond the oncogenic role of the abovementioned species, the CRC-associated microbiome has emerged as a potential screening tool as well as a prognostic and predictive biomarker. The detection of a specific bacterial signature (including *Peptostreptococcus stomatis, Parvimonas* spp., and *Porphyromonas* sp., among others) in stools may be used for screening purposes on the basis of the results of two meta-analysis of seven and eight datasets whose patients belonged to different geographic areas, including Europe, Asia, and North America [96,97]. Higher levels of *F. nucleatum* in CRC tissue correlated with worse disease-specific survival in the largest series with more than 10 years of follow-up [41]. The persistence of this same species in tumor tissue after neoadjuvant chemoradiotherapy for locally advanced rectal cancer was associated with higher relapse rates, while other studies have shown a correlation between higher levels of the bacteria and resistance to oxaliplatin and 5-fluorouracil in the adjuvant setting [12,42,43].

Beyond bacteria, the composition of other microbiota such as viruses, fungi, and archaea seem to be different in CRC but their direct impact in CRC carcinogenesis or their utility in tumor management are still unknown [46–48].

2.4. Genitourinary Cancers

Genitourinary cancers are a miscellany of tumors whose data regarding their tumor-associated microbiome is scarcer than in other malignancies. Like stool in CRC, urine must also be considered in the study of microbiome associated with kidney cancer and urothelial carcinoma. In spite of the postulated sterility of urine, very preliminary data obtained through sequencing methods suggest the presence of bacteria in the urine of healthy individuals [98].

A few studies have shown differential urine microbiome composition in patients with urothelial carcinoma when compared to healthy controls, mainly characterized by an enrichment of *Fusobacterium* and Firmicutes and a decrease of *Streptococcus* RA [49,50]. However, the potential causality relationship between the bladder tissue/urine microbiome and urothelial carcinoma—most frequent histology in bladder cancer—is yet to be elucidated. The only exception is schistosomiasis as a well-established cause of the squamous carcinoma of the bladder, but as a result of previous infection by this pathogen [99]. In renal cell carcinoma, different taxonomic profiles consistent in higher RA of *Chloroplast* and *Streptophyta* have been described in the tumor niche when compared to surrounding normal tissue [51]. A prognostic role of urine/tissue microbiome has not been described, neither in urothelial carcinoma nor in kidney cancer.

In prostate adenocarcinoma, intratumor bacteria such as *Listeria monocytogenes* have been found to be inversely correlated with adverse prognostic features (Tumor-Node-Metastasis classification, Gleason score, prostate serum antigen,levels, or androgen receptor expression) and it is hypothesized that they counteract tumor growth via local recruitment of immune cells [53]. Moreover, some other intratumor bacteria seem to correlate with specific genomic alterations associated with tumor progression and local immune suppression. From a therapeutic perspective, *Akkermansia muciniphila* seems to be relevant for the activity of abiraterone acetate in patients with castrate-resistant prostate cancer. This bacterium triggers the bacterial biosynthesis of vitamin K2, which inhibits androgen-dependent tumor growth [52].

The contribution of genital tract microbiome in the pathogenesis of female genital-tract malignancies is also raising interest. Ovarian cancer tissue samples associate a specific microbiome profile of fungi, viruses, parasites, and bacteria [100]. In the same way, endometrial cancer shows higher representation of *Porphyromonas* sp. and *Atopobium vaginae* compared with healthy tissue [54].

2.5. Other Cancers

HPV- and EBV-related cancers: both HPV and EBV are known to initiate the oncogenic process through viral DNA integration into the human genome and through acquisition of cell survival capabilities, causing different tumors depending on the body compartment or organ infected [64,101]. HPV is a well-established cause of oropharyngeal and anogenital tract squamous cell carcinomas, while EBV is directly related with nasopharyngeal and gastric cancers as well as some types of lymphoma [60,101–103]. Beyond the etiopathogenic role of these agents, they also seem to impact the composition of the tumor-associated microbiome. The group of Guerrero-Preston reported different prevalence and RA of specific taxa between HPV-related and unrelated oropharyngeal carcinomas [32]. Interestingly, this study also observed that the saliva of patients with HPV-related oropharyngeal carcinomas was found to be enriched in commensal species (*Lactobacillus* species) from the vaginal flora. In this regard, changes in the composition of the vaginal microbiome have been associated with the risk and clearance of HPV infection as well as with development of pre-malignant cervical lesions [59]. However, the mechanisms involved in these correlations have not been elucidated. Beyond vaginal fluid, stool samples from patients with localized cervical cancer showed different microbiome composition when compared to healthy controls. This brings about the possibility of using stools as a diagnostic tool for early-stage cervical cancer and, in fact, preliminary data have shown good performance in differentiating healthy patients from cancer patients according to gut microbiome profile using stool samples [104].

Data on the role of microbiome in EBV-related cancers are scarce. EBV-associated gastric carcinomas account for nearly 10% of gastric cancers [101]. A recent study involving a very small number of patients was able to detect differences in gut microbiome composition between EBV-related and unrelated carcinomas [61]. The gut bacterial functional pathways using the Kyoto Encyclopedia of Genes and Genomes data and tumor expression of immune-lipid metabolism functional proteins by immunohistochemistry (IHC) differed in terms of EBV presence as well. A score based on these factors was found to be predictive of outcome in this cancer [61]. Whether tumor-associated microbiome has a prognostic or predictive role in terms of response to therapies has not yet been evaluated.

Breast ductal carcinomas have different microbiome composition when compared to adjacent normal tissue and overlying skin within the same patient, and also when compared to breast tissue from healthy individuals [58,105]. Interestingly, intratumor taxonomic composition of breast cancer patients appear to differ also according to the tumor subtype (triple-negative vs. triple-positive ductal carcinomas).

In **lung cancer**, many studies have consistently reported different bacterial communities in the lung tissue of patients with lung cancer when compared to healthy individuals [22,106,107]. A meta-analysis of epidemiologic studies analyzed previous lung infections as risk factors for lung cancer. The results showed that a previous infection by *Chlamydia pneumoniae* or *Mycobacterium tuberculosis* was associated with an increased risk of lung cancer [55]. Although the potential mechanisms between the microbiome and lung carcinogenesis are not well-known, it seems that the metabolites produced by certain bacteria might be potentially oncogenic [56]. In that sense, pre-clinical in vitro and in vivo research from Tsay et al. showed that exposure to *Veillonella*, *Prevotella*, and *Streptococcus* bacteria are capable of inducing epithelial cell transformation through the activation of the PI3K and ERK pathways [56].

3. Microbiome and Antitumor Immunity

3.1. Interplay between the Microbiome, the Immune System, and Response to Anticancer Therapies

The crosstalk between gut microbiome and the immune system is key to maintain the intestinal homeostasis as it enables tolerance to commensal microorganisms while

inducing inflammatory responses against invading pathogens. These gut microbiome interactions are in fact crucial for shaping and modulating innate and adaptive immune responses locally and also systemically, as they are responsible for the development and maturation of myeloid and lymphoid cells [108–111]. Gut microbial communities can balance immune responses towards an anti- or pro-inflammatory effect, depending on the type of immune cell they affect [112]—specific bacteria and their by-products (metabolites) have anti-inflammatory effects by inducing T regulatory cell differentiation [113–115], while other are pro-inflammatory as they activate/stimulate dendritic cells (DC), T helper cells, or CD8+ cells [116–120]. Multiple mechanisms orchestrate this microbiome–immune system crosstalk [121]. For example, microbial-associated molecular patterns (MAMPs) from gut bacteria are detected by toll-like receptors (TLR) and can directly modify the function and maturation of innate immune cells [122]. Additionally, metabolites produced by certain bacteria such as trimethylamine N-oxide (TMAO) [123] and butyrate [124] can modulate innate immune cell differentiation and polarization [121]. Hence, the gut microbiome not only contributes to the immune system development, but also balances pro- and anti-inflammatory immune cell responses, ultimately having an effect on a variety of diseases such as cancer, auto-immune diseases, and obesity [125].

The interplay between the gut microbiome and the immune system can also affect antitumor immune-mediated responses (Figure 1) [109]. Accumulating data indicates that tumor responses to chemotherapies such as gemcitabine [38] and cyclophosphamide [126] depend on the gut microbiome. Several studies have shown a correlation between the gut microbiome composition and diversity and the efficacy of immunotherapy in patients with different tumor types, including melanoma, renal clear cell carcinoma, and lung cancer [4,15,17,127–130]. Recent data from melanoma patients revealed that the administration of stools from responders to immune checkpoint inhibitors (ICI) to non-responders can revert the primary resistance to these agents and lead to increased tumor infiltration by CD8 T cells [131], as previously suggested in pre-clinical studies [127]. Although further research is warranted, these data indicate an existing link between gut microbiome composition and tumor immune responses in cancer patients. Although the underlying mechanisms explaining this correlation are still not fully understood, a few hypotheses have been suggested [132,133]. One of the hypotheses is that some antigens are shared between bacteria and tumors and thus lead to cross-reactive T cells against the tumor cells. In this regard, recent data involving non-small cell lung cancer (NSCLC) and renal cell carcinoma (RCC) patients showed that the expression of an enterococcal cross-reactive antigen by tumors correlated with response to anti-PD-1 therapy [134]. Other proposed mechanisms include T cell priming and activation mediated by dendritic cells upon presentation of microbe- and pathogen-associated molecular patterns (MAMPs and PAMPs, respectively) present in the gut or from systemic circulation or increased pro-inflammatory cytokines and microbial metabolites [16,133,135–138]. Zhang et al. recently demonstrated that gut bacteria induce the expression of immunosuppressive chemokines in hepatocytes that cause the accumulation of MDSCs, ultimately promoting the development and growth of cholangiocarcinomas [83].

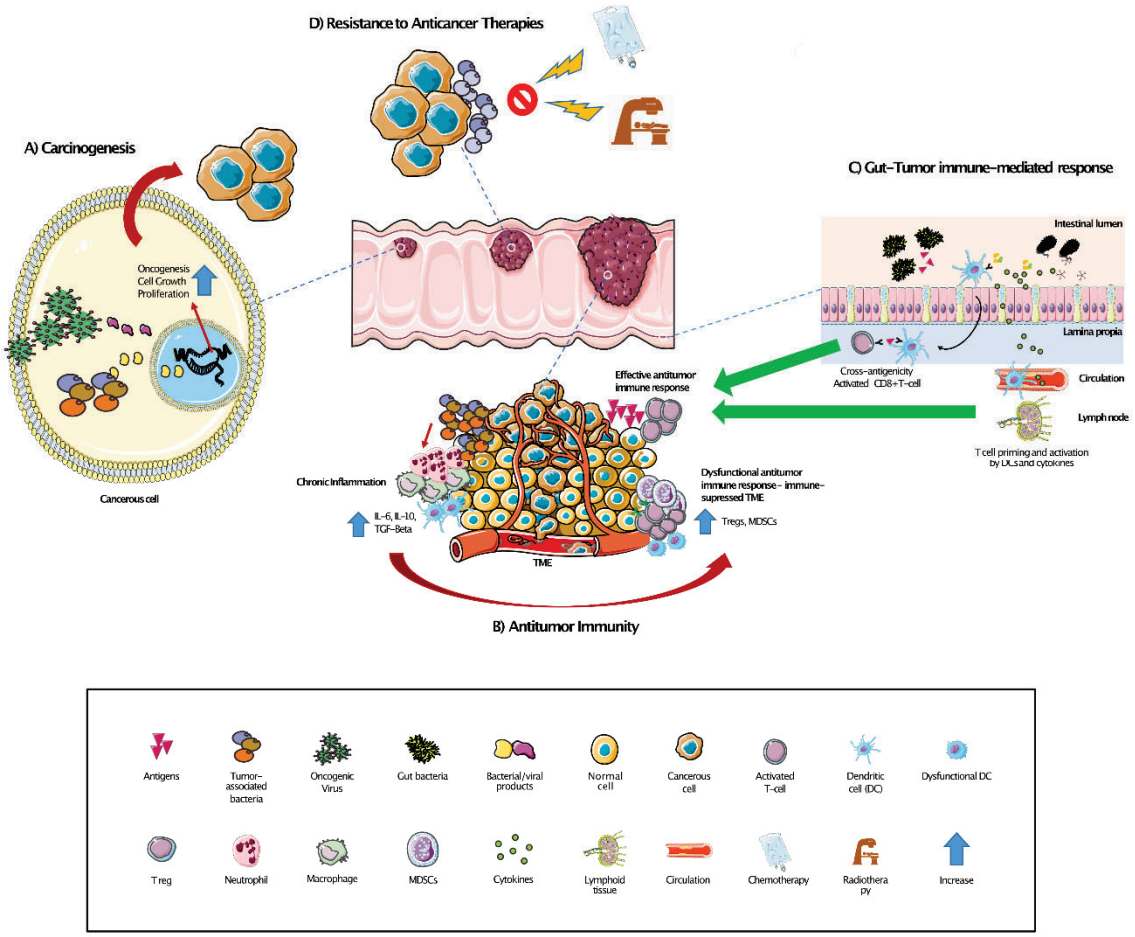

Figure 1. Impact of tumor-associated and gut microbiomes in cancer. (**A**) Carcinogenesis: intratumor bacteria and/or viruses and their by-products can activate oncogenic pathways and promote cell growth and proliferation. (**B**) Antitumor immunity: chronic inflammation caused by the local microbiome could lead to an immunosuppressive tumor microenvironment through altered antigen presentation and Tregs and myeloid-derived immunosuppressive cell (MDSC) stimulation, ultimately impairing anti-tumor immune-responses. (**C**) Gut–tumor immune-mediated response: gut bacteria and their by-products can enhance CD8+ T cell-mediated antitumor responses via (1) cross-reactivity of shared bacteria and tumor antigens recognized by T cells in the gut; (2) activation of dendritic cells, which will lead to T cell priming and expansion; (3) local pro-inflammatory cytokines or other bacterial products entering systemic circulation along with activated T cells. (**D**) Resistance to anticancer therapies: intratumoral bacteria can alter the efficacy of certain chemotherapies by altering the metabolism or through generating resistance to radiotherapy through hypoxic mechanisms.

Beyond the gut, the tumor-associated microbiome might also play a role in antitumor immune responses, although less data are available in this regard [13,38,85]. Unraveling the exact mechanisms through which gut- and tumor-associated microbiome can mediate antitumor immune-responses will be crucial in order to tailor microbiome manipulation to boost antitumor responses in cancer patients.

3.2. Modulation of Gut Microbiome to Boost Antitumor Responses

Preclinical and clinical studies strongly support the key role of the gut microbiome in the modulation of systemic and antitumor immune responses in cancer patients [139]. However, many host intrinsic and extrinsic factors such as genetic susceptibility, dietary habits, or concurrent medication contribute to the microbiome composition and diversity and might ultimately affect immune-mediated antitumor responses [140,141]. For exam-

ple, antibiotics are a known cause of gut dysbiosis [4], and their use seems to detrimentally impact on the overall survival and progression-free survival of cancer patients [142–144], and also impair responses to ICI [145,146].

The therapeutic manipulation of the gut microbiome to increase the efficacy of anticancer therapies, particularly of immunotherapy, is under evaluation, and several strategies have been proposed including dietary modifications; the use of probiotics, prebiotics, or selected antibiotics; and fecal microbiota transplantation (FMT) [18,147,148]. A recent review on this specific topic discusses the advantages and disadvantages of each of these approaches and highlights some on-going trials [149].

Dietary changes such as including or excluding specific nutrients classes (e.g., lipids) or diet supplementation with oral probiotics or prebiotics are capable of altering the gut microbiome composition [150]. Probiotics are "live organisms that might confer a health benefit to the host" while prebiotics are dietary fibers that are non-digestible by the host but digestible by gut microbes, and as such, they can favor the colonization and expansion of particular bacteria and their specific metabolites [151]. The combination of prebiotics and probiotics is known as synbiotics [152]. Pre-clinical studies suggest that diet and pre- and probiotics can enhance immune response and have antitumor properties via several mechanisms including modulation of apoptosis and cell differentiation, production of pro-inflammatory cytokines (IL-2, IL-12, and IFN-y), antioxidants (superoxide dismutase, catalase, glutathione peroxidase), and anti-angiogenic factors and reduction of cancer-specific proteins, polyamine contents, and pro carcinogenic enzymes [153,154]. However, whether they actually may enhance antitumor responses and boost the efficacy of therapies in cancer patients is still unknown.

Other strategies such as FMT, that is, a fecal suspension into the digestive tract, or stool substitutes such as oral bacterial consortia (mixture of pure live cultures of bacteria, often isolated from a stool sample of a healthy donor) are promising [155]. FMT has been proven successful for recurrent and refractory *Clostridium difficile* infection and has rapidly expanded to multiple fields of extra-gastrointestinal diseases [156,157]. Recently, Baruch et al. performed a phase I clinical trial to assess the safety, feasibility, and immune cell impact of FMT plus anti-P-D1 in PD-1 in refractory metastatic melanoma patients. Interestingly, this combination appeared safe and induced radiological tumor responses and tumor immune infiltration by CD8+T cells [131].

4. Microbiome in Oncology: Are We Ready for Prime Time?

A recent report from the International Agency for Cancer Research points out a high degree of heterogeneity across microbiome studies in terms of method of description, techniques used, taxonomic deepness, and lack of information about confounding factors [22]. There is an urge for standardization of methodology and result-reporting as well as for bias control in microbiome-related studies. Figure 2 summarizes the current challenges of microbiome studies in cancer.

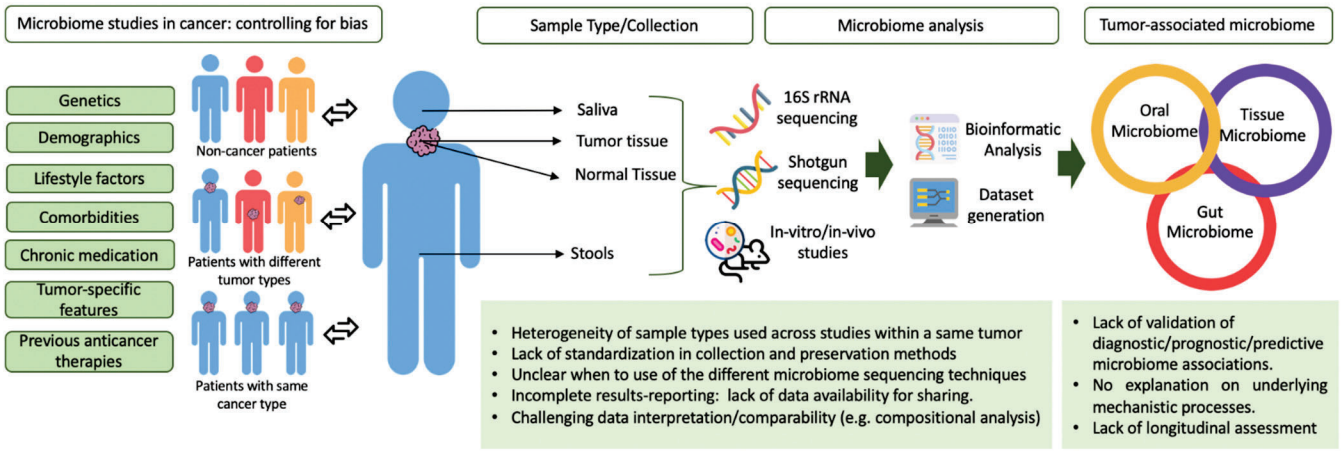

Figure 2. Challenges of microbiome studies in cancer.

4.1. Benchmarks in Standardization of Collection and Preservation Methods

Microbiome analyses can be performed in multiple types of biological samples (e.g., tumor tissue, body fluids, or stools) and using different collection and preservation methods, and as such, results obtained might vary. For instance, while gut bacterial communities seem to have a homogeneous distribution along the colon mucosa, the overall diversity of the microbiome differs when we use stool samples versus intestinal mucosal tissue [158]. The choice of sample type and collection and storage methods when studying tumor-associated microbiome is highly relevant. While it might be obvious that microbiome composition will differ between separated body compartments (e.g., oral vs urinary tract), it is unclear whether tumor tissue or a sample from the cancer-associated compartment (e.g., oral cancer tissue vs. saliva or CRC tissue vs. stool) would be equally representative. Stool samples currently used for gut microbiome analysis are limited if the goal is to study CRC-associated microbial communities [159]. In contrast, in HNC studies, microbiome composition, and diversity appeared similar when using saliva, tumor tissue, or tumor swab [33]. The same has been shown in patients with urothelial cancer when using urine and tumor tissue [160]. However, more studies to further evaluate this are needed.

Sample handling and preservation methods are relevant in order to avoid bacterial continuous growth and contamination. Several studies have analyzed the variability and the stability of microbiome diversity and composition when using different times and/or preservation temperatures [161]. In general, immediate sample freezing at -20ºC is considered the best option, but this may not be always feasible. In regard to stool samples, preservation using 95% ethanol, fecal occult blood test (FOBT), fecal immunochemical tests (FIT) tubes, Flinders Technology Associates (FTA) cards, or RNAlater provide good stability at room temperature up to 7 days, showing good correlation with fresh frozen samples [162]. No preservation media or 70% ethanol are not recommended. The International Human Microbiome Standards (IHMS) consortium (http://www.microbiome-standards.org) has published guidelines and standard operating procedures for sample collection according to the possibility to process the samples within 4 or 24h and to the possibility to freeze the sample and transport it frozen. If transcriptomic analyses are required, RNAlater can be used, having been successfully used to preserve stool and saliva samples for transcriptomic analyses, although it may impact of DNA yield [163,164].

For large-scale epidemiological studies, samples collected during CRC screening for the fecal occult blood test have been used successfully, and no major degradation of bacterial DNA has been observed. Validation studies have shown that the collection kits kept at room temperature maintain stable results up to 14 days when compared to immediately frozen samples [165].

4.2. Microbiome Analysis

Even after the microbial genetic material has been extracted, there are many technologies and techniques available for sequencing and bioinformatic analysis, each with advantages and shortcomings (Table 2). Not only have we not reached standardization of methodology, but the human microbiome itself still remains partially unknown, with different levels of "dark matter" [166].

Table 2. Methodology for microbiome analysis: problems and solutions.

Type	Technique	Problem	Solution/Alternative
Sequencing technique	16S rRNA-seq	Low taxonomic resolution Limited functional analysis	Full-length 16S sequencing, shotgun sequencing
	Whole shotgun sequencing	More expensive Human DNA also gets sequenced	Sequencing at low coverage Adequate source material, enrichment of microbial material before sequencing
	Long read sequencing	Sequencing errors are difficult to detect	Combining long read sequencing with short read shotgun
16S bioinformatics	OTU-based methods	Loss of information in clustering	ASV-based methods
	ASV-based methods	Reliance on the algorithm to detect sequencing errors	
Shotgun bioinformatics	Taxonomic profiling	Reliance on incomplete databases	New assemblies will provide more complete databases
	Functional profiling	Reliance on incomplete databases, proteins of unknown function	Further characterization of microbial proteins is still needed
	De novo assembly	Incomplete assemblies, chimeric genomes, strain heterogeneity	Strict quality control Long-read sequencing will provide better assemblies
Biostatistics	Traditional statistics	Datasets are compositional	Compositional methods, estimation of total microbial presence to avoid compositionality
	Compositional analysis	Presence of zeroes Difficult to interpret	Zero-replacement
Spatial in situ resolution	RNA in situ hybridization	Low-throughput (only 2-3 bacterium can be detected)	Use it when information about spatial resolution is needed

4.2.1. Sequencing Techniques

The most widely used sequencing techniques to perform microbiome analysis and characterize community composition (taxonomic relative abundance) in human samples are high throughput 16S ribosomal RNA gene amplicon sequencing (16S rRNAseq) and whole shotgun metagenomics [166]. 16S rRNAseq is based on amplifying the 16S rRNA gene of bacteria by PCR before sequencing, allowing for a cheap characterization of the microbiome. Usually, a few variable regions of the 16S rRNA gene are sequenced (V3–V4), providing a resolution limited to the genus level [167]. The 16S rRNA can also be sequenced with long reads, expanding the whole gene, providing higher resolution [168]. Whole shotgun metagenomics, on the other hand, is based on sequencing the whole DNA present in samples and allows for the identification of species and genes of all microorganisms, not only of bacteria, provided that sequencing depth is adequate. In the case of 16S sequencing, the PCR amplification step guarantees that only microbial DNA

will be sequenced. This is not the case for shotgun sequencing, where the DNA samples need to be enriched for microbial DNA beforehand.

4.2.2. Bioinformatic Analysis

Bioinformatics analysis of 16S samples has traditionally relied on clustering similar sequencing reads up to a level of similarity (normally, 97%) into operational taxonomic units (OTUs). This clustering removes sequencing errors, but this also implies a loss of information. Alternatively, novel approaches attempt to retain all amplicon sequence variants (ASV). As opposed to clustering reads, they attempt to algorithmically distinguish sequencing errors from biological variation [169,170]. QIIME2 is a bioinformatics toolkit that provides frameworks for integrating all steps of 16S analysis [171].

In the case of shotgun metagenomics, many analysis lines are available. On one hand, read-based classification algorithms aim to provide a taxonomic assignment to each sequencing read. Taxonomic profiles allow us to analyze which microorganisms are present in biological samples, qualitatively and/or quantitatively. Different software implementations are available for this task [172]. Reads can also be classified by functional potential as opposed to taxonomy. These algorithms classify reads into gene families, which can provide different insights (identification of toxicity genes, reconstruction of pathways, etc.). In both cases, what can be detectable is limited to what is present in the databases that are used. Lastly, shotgun metagenomics reads can be used to reconstruct the original genomes (de novo assembly). This approach does not rely on any database, and thus it can be used to discover new genomes. For a review of bioinformatics methodology for shotgun metagenomics, please see the study by Breitwieser et al. [173].

4.2.3. Statistics for Microbiome Analysis

Calculation of diversity metrics is common when analyzing taxonomic profiles. Diversity measures (Shannon, Simpson indices) are used to query the within-sample diversity or diversity, while β diversity metrics (Bray–Curtis, UniFrac) are used to investigate between-sample diversity. Besides diversity analyses, common statistics may be used to find statistically significant differences between groups. However, it is important to note that sequencing-derived microbiome datasets are compositional**,** that is, they do not provide absolute descriptions of the microbiome, but are relative to the whole microbiome present in each sample, requiring specific statistical methodology [174]. This complicates the interpretation of results and arises the possibility of spurious associations unless specific methodology is used. Alternatively, quantification of the total microbial load completely avoids the problem of compositionality and has been shown to provide more insights [175].

4.2.4. Spatial In Situ Resolution

Although metagenomic and metatranscriptomic analyses have revolutionized the study of microbial communities, they have the main drawback of not providing spatial information on how these communities are distributed in the sample, thus preventing a full understanding of how the bacteria interact with each other or with the microenvironment [176,177]. Fluorescence in situ hybridization (FISH) targeting the rRNA can identify almost any microbe in a given tissue sample [178]. Fluorescence spectral imaging allows for the differentiation of many fluorophores identifying all members of a complex microbial community, thus offering a systems-level view of the spatial structure of the microbiome [179]. Because of technical limitations, rRNA FISH can only be used to differentiate only two or three microbial types simultaneously.

RNAscope is a recently developed RNA in situ hybridization technology that allows for direct visualization of RNA in formalin-fixed, paraffin-embedded (FFPE) tissue, enabling sensitive and specific spatial analysis of all RNA molecules present in a sample simultaneously [180]. In a study conducted by Serna et al., RNAscope technology was

used to visualize *F. nucleatum* in rectal cancer tissue and to evaluate how this species [42] interacts with host cells within the tumor microenvironment. An automated version of the RNA in situ hybridization assay was originally developed for bacteria visualization in matched primary and metastatic CRC-intact FFPE tissues [94].

4.2.5. Pre-Clinical Tools to Study Microbiome in Cancer

In vivo models are needed to understand the mechanisms through which some microbial communities or specific single microorganisms drive tumorigenesis [181]. Murine models provide excellent tools to study microbiota-associated human diseases [182]. Two main methods have emerged to explore the effects of the microbiota on physiology and disease in mice: germ-free models, which can be used for studying the functional properties of microbiome, and broad-spectrum antibiotic-treated models, which are used to study the cause–effect relationship between dysbiosis and resistance to therapies.

Beyond in vivo studies, in vitro models are also required to study the complexity of microbial interactions as they have the advantage of not being influenced by factors such as age, sex, diet, geography, genetic background, and antibiotic use, which may lead to bias in human and animal models [183]. Examples of in vitro models include organoids cultures and bioreactor system. The organoids are a three-dimensional culture of tissue that represent an excellent system for studying how microbiota induces and promotes cancer growth [184]. These technologies can be used to investigate the impact of dysbiosis on tumorigenesis and to find therapeutic strategies to modulate the microbiome to improve treatment efficacy [185]. For instance, the use of organoids in a study evaluating the role of *Helicobacter pylori* in gastric carcinogenesis was able to demonstrate how this species promoted cell proliferation and activation of the *c-Met* oncogene through NF-κB signaling [186].

Another in vitro model is the Bioreactor system, which allows for the study of complex gut microbial ecosystems in a controlled environment [187]. The "Robogut" bioreactor has been established in the Allen-Vercoe laboratory to culture gut microbial ecosystems in vitro under physiologically relevant conditions [188]. The laboratory uses the bioreactors as a model of the colonic microbiota in determining the effectiveness of antibiotic pretreatment in ulcerative colitis caused by *Clostridioides* [189]. The goal of this technology is to culture novel and highly fastidious species that cannot be cultured using conventional methods of cell culture in static dishes [190].

4.3. Challenges in Microbiome Studies in Cancer: Controlling for Bias

Observational studies of the intra-tumoral microbiome can be broadly grouped into (1) those with the goal of evaluating tissue microbiome composition in relation to prognostic events (treatment response/resistance, tumor recurrence, and tumor-related mortality) in patients with cancer or precursor lesions (case-only studies), and (2) those with the goal of comparing tissue microbiome composition between patients with cancer (or precursor lesions) and individuals free of cancer (case–control studies). A key component of group 1 studies is the evaluation of prognostic events over time. As in all clinical or epidemiological studies of the microbiome, other sources of microbiome variation need to be accounted for in the statistical analysis if they act as confounders or modifiers of the association between microbiome composition and prognostic events. Depending upon the tumor type, these could include clinical features of the diagnosed tumors (e.g., diagnostic method and stage, previous surgeries, familial gene mutations, other genetic variants, and diagnostic or prognostic biomarker levels), recent usage of pharmaceutical drugs (e.g., antibiotics, proton-pump inhibitors, metformin, and non-steroidal anti-inflammatories), demographic factors (e.g., place of residence, age, sex, and race/ethnicity), and lifestyle factors (e.g., diet or nutritional status at diagnosis, body weight, tobacco smoking, and alcohol consumption). Measurement errors in assessing lifestyle factors, especially dietary intake, must be carefully considered when planning a study. Nutritional status at diagnosis or surgery could be an alternative if an accurate

dietary assessment by questionnaire is not available or feasible. Ideally, tissue specimens for microbiome analysis should be collected before therapeutic interventions, but this may not be possible if neoadjuvant chemo- or radiotherapy is indicated. In general, lifestyle and demographic factors have not shown strong associations with microbiome composition in terms of stool samples, but it should be noted that these associations remain largely unexplored in studies of tissue-specific or organ-specific microbiomes [191].

In group 2 studies, tissue microbiome composition is compared between cancer patients and cancer-free individuals—these are essentially case–control studies; moreover, in the absence of major biases due to study execution including recruitment strategy, microbiome analysis process, or differential errors in the measurement of epidemiologic variables, such studies would have the intention to evaluate tissue microbiome composition as a potential risk factor for the development of cancer [192]. In such studies, tissue samples collected for microbiome assessment are usually measured at, or shortly after, the date of diagnosis, and this fact is a major weakness of this study design since observed associations may not be causal. Further, depending upon the tumor site, the acquisition of normal tissues from cancer-free individuals from the same base population as the cases could range from moderately challenging to impossible. Normal colonic mucosal tissue from individuals free of cancer can be relatively easily obtained in studies using tissue collection via colonoscopy. Studies that utilize normal tissue obtained from national tissue banks or local tissue donor programs might be useful in giving a general overview of microbiome composition in individuals free of cancer, but caution should be exercised when interpreting differences with tumor tissues since tissue-bank or donor normal tissues may differ in other important ways such as age, overall health status, and other variables. The study of case–control differences in oral or gut microbiome composition from saliva or stool samples as proxies for tissue microbiomes or as risk factors themselves (e.g., via systemic effects on inflammation) has been considered in the majority of epidemiological studies of microbiome composition as a potential risk factor for cancer [22].

It is important to note that in the absence of a truly prospective cohort study in which biological samples for microbiome composition are collected before disease onset, microbiome-disease association signals observed in retrospective case–control studies may or may not be causal. Replication of observed association signals in additional case–control studies from similar and different populations is therefore necessary, as well as deeper mechanistic investigation through in vivo studies, for instance [191].

5. Future Directions

The study of microbiome as a new hallmark of cancer is just getting started. In this past year, 2048 publications related to "microbiome AND cancer" were indexed in PubMed, nearly 2000 more than 10 years ago. Whether understanding the tumor-associated microbiome will lead to a better comprehension of the pathogenesis of disease and corresponding molecular traits and will ultimately become a clinically useful biomarker tool for cancer prevention, diagnosis, and treatment is yet to be fully established, although evidence for this is beginning to accumulate. Examples of that are the microbiome-based screening tests for early detection of CRC, or the encouraging results of a phase I trial using FMT to boost ICI responses in refractory melanoma patients [131,193].

Despite the amount of knowledge being gathered, there are still some caveats that should be addressed. One of the most urgent is the standardization of microbiome methodology from sample collection to bioinformatic analysis in order to improve comparability/interpretation of results across studies [194]. Initiatives such as The Microbiome Quality Control (MBQC) project are already working to overcome this challenge. Special focus should be put on unveiling mechanistic processes to better define the link between microbiome (tumor-associated or from compartments distant from tumor-hosted organ) and carcinogenesis. More preclinical and clinical studies are needed

to evaluate not only the community composition but also associated functional and multi-omic analyses. The Human Microbiome Project Consortium found shared metabolic pathways between healthy individuals despite having different microbiome taxa composition, which could also be the case in cancer patients [195]. Overall, there is a lack of longitudinal studies assessing the potential evolution of the microbiomes relevant for cancer. Both the microbiome and tumorigenesis are dynamic "systems". Although viral and bacterial genomes appear to be stable in time in healthy individuals, point mutations in some bacteria could lead to a functional change, such as antibiotic resistance [195–198]. In addition, changes in extrinsic factors can also cause microbiome compositional variations over time and impact the results of microbiome manipulation strategies. Currently, different therapeutic strategies are under evaluation in clinical trials. Solving the knowledge gaps and the abovementioned weaknesses will allow clinicians to better determine who might benefit the most from these therapies. In fact, several questions remain to be answered regarding the use of microbiome therapeutics such as best approach or setting (in combination with standard chemo-, radio-, or immunotherapy, in metastatic or adjuvant settings), potential toxicities, ethical implications, and classification [3]. Of note, some of these therapies such as prebiotics or probiotics are widely used in the general population without proper regulation [199].

Microbiome research in oncology is an exciting field to be explored. The creation of collaborative multidisciplinary networks will be fundamental to augment the knowledge and optimize resources. Continued efforts should be made to overcome the challenges and ensure that we are ready for prime time.

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ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

ANNALS OF ONCOLOGY driving innovation in oncology

ORIGINAL ARTICLE

First-in-class Microbial Ecosystem Therapeutic 4 (MET4) in combination with immune checkpoint inhibitors in patients with advanced solid tumors (MET4-IO trial)

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Background: The intestinal microbiome has been associated with response to immune checkpoint inhibitors (ICIs) in humans and causally implicated in ICI responsiveness in animal models. Two recent human trials demonstrated that fecal microbiota transplant (FMT) from ICI responders can rescue ICI responses in refractory melanoma, but FMT has specific limitations to scaled use.

Patients and methods: We conducted an early-phase clinical trial of a cultivated, orally delivered 30-species microbial consortium (Microbial Ecosystem Therapeutic 4, MET4) designed for co-administration with ICIs as an alternative to FMT and assessed safety, tolerability and ecological responses in patients with advanced solid tumors.

Results: The trial achieved its primary safety and tolerability outcomes. There were no statistically significant differences in the primary ecological outcomes; however, differences in MET4 species relative abundance were evident after randomization that varied by patient and species. Increases in the relative abundance of several MET4 taxa, including *Enterococcus* and *Bifidobacterium*, taxa previously associated with ICI responsiveness, were observed and MET4 engraftment was associated with decreases in plasma and stool primary bile acids.

Conclusions: This trial is the first report of the use of a microbial consortium as an alternative to FMT in advanced cancer patients receiving ICI and the results justify the further development of microbial consortia as a therapeutic co-intervention for ICI treatment in cancer.

Key words: intestinal microbiome, first in class microbial ecosystem therapeutic 4, immune checkpoint inhibitors, advanced solid tumors

INTRODUCTION

The composition of the human intestinal microbiome is implicated in response to immune checkpoint inhibitor (ICI) treatment in cancer,[1-3] and consequently a target for therapeutic augmentation.[4] Fecal microbiota transplantation (FMT) is now under investigation as a co-therapy designed to augment ICI responses in multiple trials registered on ClinicalTrials.gov, including two trials with published results demonstrating rescue of ICI non-response with FMT in melanoma,[5,6] indicating a broad interest in this new modality. However, FMT has practical limitations affecting its generalizability, safety and appropriateness for use at scale.[7] Microbial consortia (multi-species mixtures of cultivated microbes) represent an intermediate approach intended to balance the ecological and functional complexity of FMT and the practical advantages of cultivated microbes, and have been successfully used as alternatives to FMT for other indications such as *Clostridioides*

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difficile infection,[8,9] including in a phase III trial in which efficacy similar to FMT was reported.[10]

Microbial Ecosystem Therapeutic 4 (MET4) is an orally delivered defined mixture of pure live cultures of intestinal bacteria isolated from the stool of a healthy donor, purified and grown in conditions modeling those of the human distal gut.[8] MET4 is composed of 30 phylogenetically and functionally diverse bacterial species including taxa previously associated with ICI responsiveness in published reports (Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2023.02.011). MET4 is cultured *in vitro* and each strain is individually characterized genotypically and phenotypically, including for antimicrobial susceptibilities. MET4-IO is a single-center investigator-initiated clinical trial designed to evaluate the safety, tolerability and engraftment of MET4 in patients with advanced solid tumors receiving ICI. This study included a safety cohort (group A) and two additional cohorts of ICI-naïve (group B) or pre-exposed (group C) patients, randomized to receive either standard-of-care ICI alone or in combination with MET4 (NCT03686202).

PATIENTS AND METHODS

Patient population

Adult patients with advanced solid malignancies with an Eastern Cooperative Oncology Group performance status of 0-2, able to swallow and receiving (groups A and C) or planned to receive (group B) standard-of-care anti-programmed cell death protein 1 (PD-1) monotherapy or anti-PD-1 plus anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) combination immunotherapy were included in the study. Multiple tumor types were enrolled. Additional eligibility criteria included measurable disease by computed tomography or magnetic resonance imaging as per RECIST v1.1 and willingness to undergo serial collection of blood and stool samples. Gastrointestinal disorders likely to interfere with absorption and prior treatment with immune checkpoint blockade in group B were key exclusion criteria (full protocol in Supplementary Material, available at https://doi.org/10.1016/j.annonc.2023.02.011).

Study design and treatment

This single-center, open-label, investigator-initiated study initially included three cohorts of patients (groups A, B and C). In group A (safety cohort), MET4 was added to standard-of-care anti-PD-1 antibody until unacceptable toxicity or progression. Upon completion of group A, groups B and C were opened to enrollment. In group B, eligible subjects with advanced solid tumors naïve to ICI were randomized in a 3 : 1 ratio to receive MET4 in combination with ICI (experimental arm) or ICI alone (control arm) with a run-in period of ICI therapy (one cycle). In group B, patients could be treated beyond progression provided they had a clinical benefit without clinical deterioration and did not have substantial adverse effects, as assessed by the investigator. In group C, eligible subjects with advanced solid tumors already on treatment with standard-of-care ICI with first unconfirmed progression on evaluation scans, clinically stable and suitable to be treated beyond progression as per investigator's assessment were randomized in a 1 : 1 ratio to receive MET4 in addition to ICI inhibitor (experimental arm) or continue with ICI alone (control arm) (Supplementary Figure S1A, available at https://doi.org/10.1016/j.annonc.2023.02.011). The protocol was amended to include group D, designed to evaluate MET4 in high-risk melanoma patients on adjuvant immunotherapy. Results of group D will be reported separately once accrual is completed.

In groups A, B and C, MET4 capsules were administered orally with an initial loading dose of 20 capsules (2-10 $\times$ 10^{10} colony-forming units) over 2 days, followed by a maintenance dose of 3 capsules (6-30 $\times$ 10^{9} colony-forming units) continuous daily dosing for a total of 1 year or until unacceptable toxicity, progression of disease or discontinuation of treatment for any cause. Standard-of-care ICI was dependent on tumor type and included single-agent nivolumab (480 mg flat dose q4w), or pembrolizumab (200 mg flat dose q3w), or nivolumab (360 mg flat dose q3w) in combination with ipilimumab (at either 3 mg/kg q3w or 1 mg/kg q3w for up to four infusions) followed by maintenance nivolumab at 480 mg flat dose q4w as per standard of care, until unacceptable toxicity, disease progression, completion of therapy based on approval indication or discontinuation for any cause.

Sample collection

In groups A and C, stool samples were collected before initiation of MET4 (screening visit: T0); day 10-16 from initiation of MET4 (T1); week 3-4 (window: $+2$ weeks) from MET4 initiation (T2); week 24 (window: ± 2 weeks) from MET4 initiation (T3); and 1-2 weeks post-end of treatment (EOT) (T4). Blood samples were collected at T0, T2 and T3, based on the same timepoint definitions.

In group B, stool samples were collected before initiation of ICI (T -1); week 3-4 post-ICI, before initiation of MET4 (window: $+2$ weeks) (T0); day 10-16 from initiation of MET4 (T1); week 3-4 (window: $+2$ weeks) from MET4 initiation (T2), week 24 (window: ± 2 weeks) from MET4 initiation (T3); and 1-2 weeks post-EOT (T4). Blood samples were collected at baseline T -1, T0, T2 and T3, based on the same timepoint definitions. Study design and timeline of sample collection are summarized in Supplementary Figure S1B, available at https://doi.org/10.1016/j.annonc.2023.02.011.

Outcome measures

Primary endpoints included cumulative relative abundance of MET4 taxa at T1, changes in relative abundance of MET4 taxa between T0 and T1 and treatment-related adverse events (AEs) assessed by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) v5.0. For the ecological co-primary endpoint, patients were considered assessable if stool samples were obtained at T0 and T1 (a total of two stool samples in groups A and C and three stool samples in group B).

Secondary endpoints included cumulative relative abundances of MET4 taxa at T2-T4, changes in relative abundance of MET4 taxa between baseline (T0) and post-randomization timepoints and bacterial taxonomic diversity between T0 and T2-T4. Exploratory outcome measures included overall response rate measured as per RECIST v1.1 and immune RECIST (iRECIST).

Study assessments

Response assessments were defined according to RECIST v1.1. Time of assessment was based on investigator evaluation and tumor type and typically occurred every 2-3 cycles of immunotherapy until disease progression or treatment discontinuation. Patients treated beyond progression were considered to have progressive disease at the time of the initial progression event, as assessed by the investigator, regardless of subsequent tumor response. Any patient who received at least one dose of MET4 was included in the assessment of safety. Patients were required to complete a study diary to assess appropriate dosing and study compliance. Reason for any missed doses of MET4 was recorded. AEs attributable to immunotherapy and MET4 were graded according to the NCI-CTCAE v5.0. Safety assessments were carried out continuously during treatment, and up to resolution or stabilization of the AEs, whichever occurred first.

Study oversight

The study protocol (Supplementary Material, available at https://doi.org/10.1016/j.annonc.2023.02.011) and all the related amendments were approved by the Institutional Review Ethics Board. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice as defined by the International Conference on Harmonization. Before enrollment, all patients provided written informed consent. Established bi-weekly safety calls occurred to provide oversight of safety. Data collection and monitoring were carried out throughout the study and after enrollment was completed. Monitoring of study conduct, including all AEs, was carried out by the Princess Margaret Cancer Centre Data Safety Monitoring Committee twice a year and as needed.

Microbiome analysis

DNA was extracted from the patients' frozen fecal material using the Quick-DNA Fecal/Soil Microbe Kits (Zymo Research, Irvine, CA) and normalized by stool weight. Library generation and next generation sequencing were done at MR DNA Molecular Research (Shallowater, TX). The 16S ribosomal RNA gene V4 variable region was amplified with PCR using primers 515F (GTGYCAGCMGCCGCGGTTA) and 806R (GGACTACNVGGGTWTCTAAT), with the barcode on the forward primer, and HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MD). PCR consisted of 30 cycles of 94°C for 3 min, then 30-35 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 60 s, and a final elongation step at 72°C for 5 min. After amplification, PCR products were resolved by electrophoresis on a 2% agarose gel to determine amplification and relative band intensity. Multiple samples were pooled in equal proportions, on the basis of their molecular weight and DNA concentrations and purified with calibrated AMPure XP beads (Beckman Coulter, Brea, CA). Pooled and purified PCR product was used to prepare an Illumina (San Diego, CA) Nextera DNA library. Sequencing was done by MR DNA using an Illumina MiSeq with version 3 reagents and generating 300-bp paired-end reads. Reads in which >70% of bases had a Phred score of 30 or more were retained and trimmed using DADA2 (v1.14.1). Taxonomy was assigned with a native implementation of the naïve Bayesian classifier method and trained with the Silva database (v132). Amplicon sequence variants were assigned and collated to the closest related taxon using NCBI BLAST.

Targeted metabolomics

Plasma and stool samples were sent to The Metabolomics Innovation Centre (TMIC) (Edmonton, Alberta, Canada) for targeted metabolomic profiling using liquid chromatography-tandem mass spectrometry as described in the Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.011. Samples were profiled for panels of bile acids (BAs) and short-chain fatty acids (SCFAs). Analytes were included in statistical analyses if they were detectable in at least 40% of samples.

Statistical analysis

Frequencies of immune-related AEs (irAEs) between MET4 recipients and controls, and single versus combination therapy ICI were compared by chi-square test. Ecological outcomes (MET4 relative abundance, change from baseline, number of taxa >1%, Shannon diversity and observed operational taxonomic units) were compared between MET4 recipients and controls as continuous variables with unpaired *t*-tests or analysis of variance (ANOVA) with post-tests. For alpha diversity metrics, samples were rarefied to a sequencing depth of 35 501 reads (the lowest depth among all the samples included in the analysis). Rarefied and unrarefied analyses were carried out and compared. Fold change in relative abundance between baseline samples (pre-MET4) and post-MET4/control exposure timepoints was generated by dividing post-treatment relative abundance by the baseline relative abundance and log transforming the resulting fold change, and then using one-sample *t*-tests to compare the distribution of these values to a 'no change' reference value of 0. Volcano plots for changes in relative abundance in taxa after randomization between MET4 recipients and controls were generated by using MaAsLin2 with study participant included as a random effect. Compositional differences in Bray—Curtis dissimilarity were plotted on principal coordinate analysis plots and compared by permutational multivariate analysis of variance (PERMANOVA).

Concentrations of metabolites were compared across sampling timepoints for MET4-treated and control randomized individuals using ANOVA with log10 transformation when appropriate. Log2-fold change (L2FC) in

metabolite concentration was calculated by dividing T2 (post-MET4) metabolite concentrations by T0 (baseline, pre-MET4) metabolite concentration and log2 transforming the data. Patients were defined as ecological responders (EcoRs) if they had at least five MET4 taxa increasing by at least log10 post-MET4 initiation. Differences between L2FC in metabolites were compared for MET4-treated patients in EcoRs and ecological non-responders (EcoNRs) by ANOVA.

All analyses were carried out in GraphPad Prism or R (San Diego, CA).

RESULTS

Study patient population

Between December 2018 and December 2020, 40 patients receiving standard-of-care monotherapy or combination ICI were enrolled. The trial profile, total population and assessable subjects are summarized in Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2023.02.011. In an initial safety cohort (group A, $n = 6$), one subject was enrolled and received one cycle of anti-PD-1 antibody within the trial. However, due to rapid disease progression, the patient never started MET4 and was replaced, for a total of five assessable patients. In group B ($n = 30$), patients were randomized 3 : 1 to the experimental arm (ICI plus MET4, $n = 22$) or control arm (ICI, $n = 8$). Accrual in group C ($n = 4$) was discontinued before enrollment was complete, due to the limited number of pseudo-progression events in patients receiving ICI, clinical deterioration at the time of disease progression and alternative treatment opportunities as a preferred strategy by both patient and physician. Baseline demographics, disease characteristics and number of previous lines of therapy are presented in Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2023.02.011. Head and neck squamous cell carcinoma (HNSCC) ($n = 20$) and melanoma ($n = 16$) were the most common tumor types. All patients (cohorts A, B and C) received anti-PD-1 antibodies and 13 (33%) received anti-PD-1 and anti-CTLA-4 antibodies in combination. Twenty-six patients ($n = 5$ in group A, $n = 19$ in group B and $n = 2$ in group C) received at least one dose of oral MET4 in combination with ICI. Patient characteristics according to HNSCC and melanoma tumor types are summarized in Supplementary Tables S3 and S4, available at https://doi.org/10.1016/j.annonc.2023.02.011, respectively.

Median follow-up duration, defined as the time from enrollment (for group A) and randomization (for groups B and C) to data cut-off (22 May 2021) or last follow-up, whichever occurred first, was 164 days (range 41-858 days) in group A, 104 days (range12-666 days) in group B and 125.5 days (range 74-259 days) in group C. Median MET4 duration of treatment was 38 days (range 0-334 days) excluding missed doses. At the time of analysis at data cut-off, three patients remained in follow-up (one in group A and two in group B) and five patients (group B) remained on treatment, three of them being in the experimental arm with MET4.

MET4 was safe and tolerable in standard-of-care ICI recipients

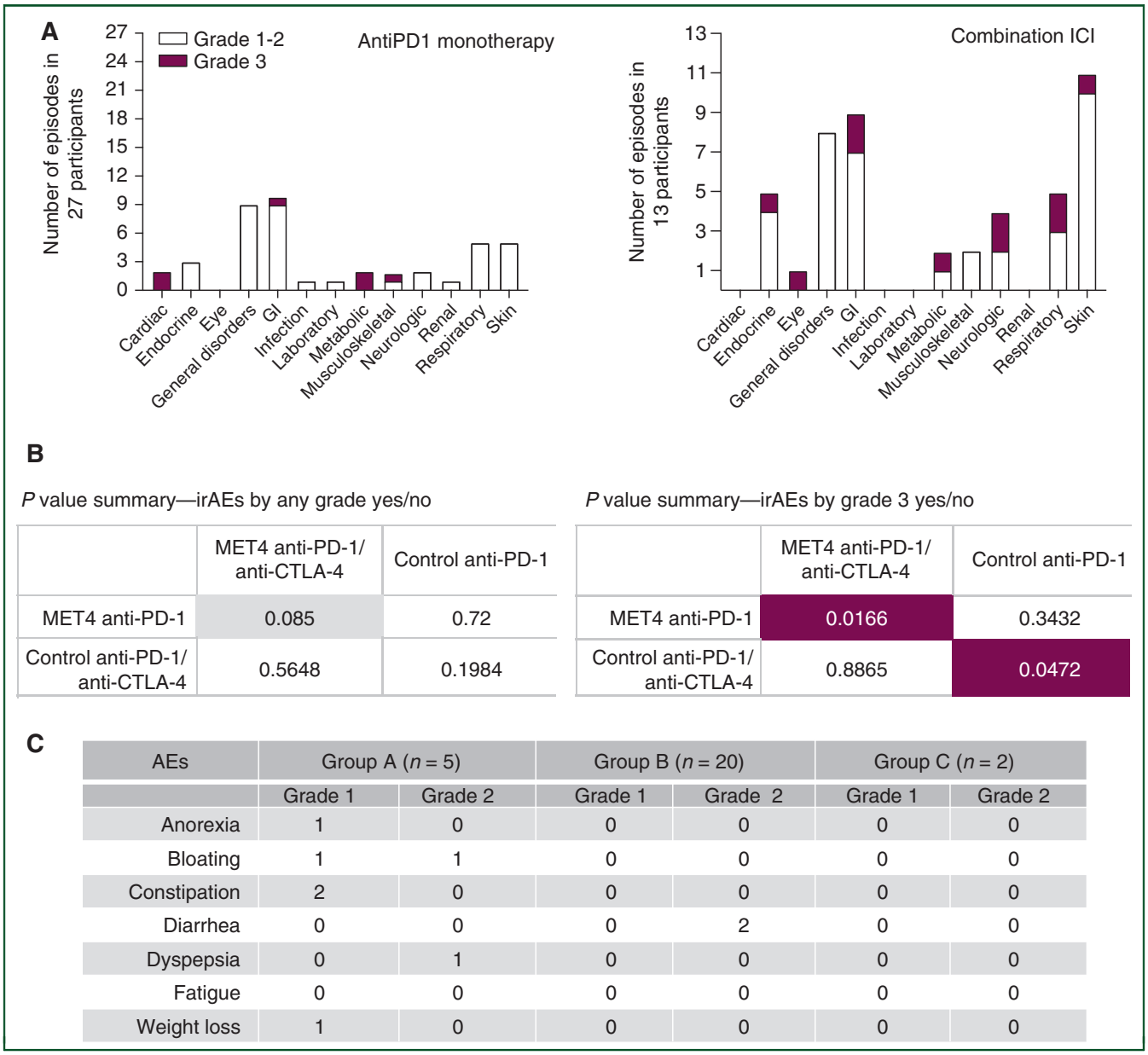
In total, 39 patients received at least one cycle of ICI and were assessable for safety analysis. The ICI-related AEs observed in our patient population were consistent with the literature with a higher frequency of severe AEs in the patients receiving anti-PD-1 with anti-CTLA-4 antibody combination (10 grade 3-4 AEs in 13 patients, 77%) as compared to single-agent anti-PD-1 antibody (6 grade 3-4 AEs in 26 patients, 23%) (Figure 1A, Supplementary Table S5, available at https://doi.org/10.1016/j.annonc.2023.02.011). There were no statistically significant differences between the MET4 and control groups with respect to the number of irAEs of any grade or grade $\geq$3 only (Figure 1B). Of the 26 patients (5 in group A, 19 in group B and 2 in group C) who received at least one dose of MET4, 10 of them were treated with anti-PD-1/anti-CTLA-4 antibody combination. Within groups A, B and C, a total of 29 patients were assigned to receive MET4 either in combination with anti-PD-1 or anti-PD-1 and anti-CTLA-4. MET4-attributed AEs occurred in 17% (5/29) of patients, were mainly gastrointestinal, mild/moderate in severity (grade 1-2) and all resolved without sequelae. No MET4-related grade $\geq$3 AEs were observed in the study population.

Treatment outcomes in MET4 recipients and controls

RECIST v1.1 best treatment response in all groups is summarized in Supplementary Table S6, available at https://doi.org/10.1016/j.annonc.2023.02.011. In the entire cohort, there were 2 patients with complete responses (1 control, 1 MET4), 7 with partial responses (PRs) (all MET4 recipients), 9 with stable disease (SD) (3 control and 6 MET4 recipients) and 17 with progressive disease (5 control, 12 MET4 recipients). Four patients were not assessable for response assessment. RECIST treatment responses for patients assessable for ecological primary outcomes and by tumor types are summarized in Supplementary Tables S7 and S8, available at https://doi.org/10.1016/j.annonc.2023.02.011, and Figure 2 (for cohort B only, which included ICI-naïve patients). The overall RECIST response rate for MET4 recipients in cohort B was 35% (6/17) versus 14% (1/7) in controls (Fisher's exact $P = 0.37$). Clinical benefit (patients with PR or SD $\geq$6 months) was observed in 53% (9/17) of MET4 recipients as compared to 20% (1/5) of patients in the control arm, $P = 0.18$. The clinical benefit could not be assessed in two of the seven patients in the control arm due to inadequate follow-up ($<$6 months).

MET4 treatment increased the number and relative abundance of administered taxa in a subset of recipients, but not across all recipients

A total of 147 stool samples were sequenced [113 from MET4 recipients and 34 from subjects treated with ICI alone (control)], of which 92 were collected after exposure to MET4 or the control intervention post-randomization (including all time points). A total of 30 patients [5 in group A, 21 in group B (15 in the experimental arm and 6 in



B

P value summary—irAEs by any grade yes/no

	MET4 anti-PD-1/ anti-CTLA-4	Control anti-PD-1
MET4 anti-PD-1	0.085	0.72
Control anti-PD-1/ anti-CTLA-4	0.5648	0.1984

P value summary—irAEs by grade 3 yes/no

	MET4 anti-PD-1/ anti-CTLA-4	Control anti-PD-1
MET4 anti-PD-1	0.0166	0.3432
Control anti-PD-1/ anti-CTLA-4	0.8865	0.0472

C

AEs	Group A ($n = 5$)		Group B ($n = 20$)		Group C ($n = 2$)	
	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2
Anorexia	1	0	0	0	0	0
Bloating	1	1	0	0	0	0
Constipation	2	0	0	0	0	0
Diarrhea	0	0	0	2	0	0
Dyspepsia	0	1	0	0	0	0
Fatigue	0	0	0	0	0	0
Weight loss	1	0	0	0	0	0

Figure 1. Immune-related and MET4-attributed AEs in the MET4-IO trial. (A) Type of irAE experienced by system for anti PD1 monotherapy and combination ICI recipients, colored by grade. Details of each system category are reported in Supplementary Table S5, available at https://doi.org/10.1016/j.annonc.2023.02.011. (B) Chi-square *P* values for comparison of irAEs between MET4 recipients and controls and combination versus single-agent ICI receipt for any AE (left) and grade 3 AEs only. (C) MET4-attributed AEs for MET4 recipients in groups A-C.

AEs, adverse events; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; GI, gastrointestinal; ICI, immune checkpoint inhibitor; irAE, immune-related adverse event; MET4, Microbial Ecosystem Therapeutic 4; PD-1, programmed cell death protein 1.

the control arm) and 4 in group C] were assessable for the ecological co-primary objective. Two patients in cohort B (B009 and B022) did not provide a stool sample at T1 window and were included only for safety/tolerability and ecological secondary outcomes. Two patients (B011 and B027) received only one dose of MET4 and were excluded from analysis of all ecological outcomes (Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2023.02.011).

The trial ecological co-primary outcomes, the relative abundance of MET4 taxa at T1 (range day 10-16) and change in relative abundance of MET4 between T0 and T1 are shown in Figure 3A and B. The mean (±standard deviation) cumulative relative abundance of MET4 taxa on T1 in the MET4 group was 0.30 ± 0.13 versus 0.22 ± 0.11 in controls ($P = 0.098$). The mean change in relative abundance of MET4 taxa in the MET4 group was an increase of 0.033 ± 0.12 versus a decrease of 0.063 ± 0.10 in the control group ($P = 0.059$). Paired analysis of pre-/post-MET4 alpha diversity and cumulative relative abundance in the stool of MET4 recipients was not significantly different. There were no differences in the secondary ecological outcomes between MET4 recipients and controls (Figure 3C-F), including the cumulative relative abundance of MET4 taxa or change in cumulative relative abundance of MET4 taxa at later timepoints, or taxonomic Shannon

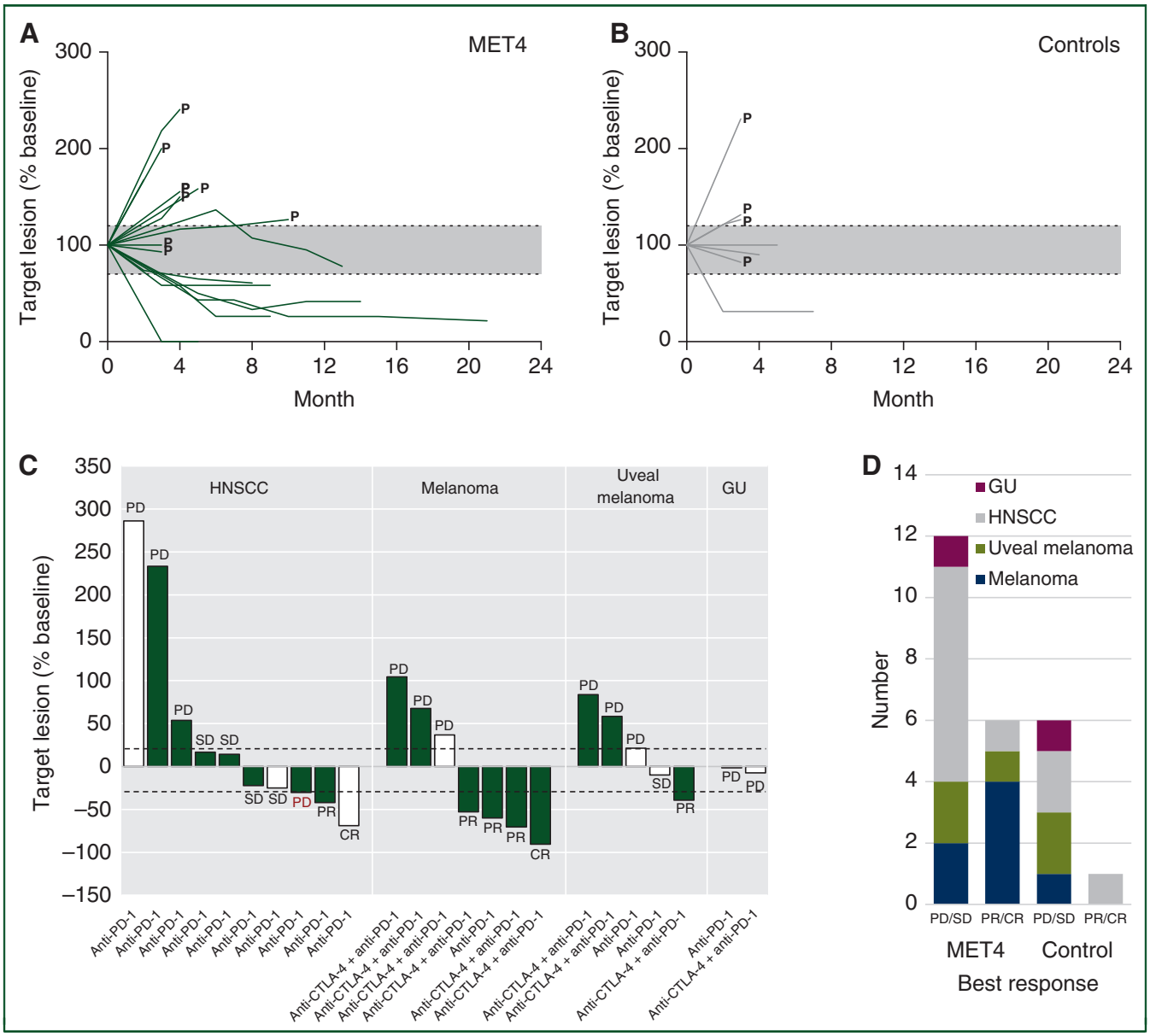


Figure 2. Therapeutic responses in study patients. (A) Patients' target lesion. Target lesion size as a percentage of baseline is shown in panels A (MET4 recipients) and B (controls, $n = 6$) for the study period for patients with evaluable tumors. *P* indicates unequivocal progression of a non-target lesion. (C) Waterfall plots indicate best response change in tumor size compared to baseline stratified by tumor type. RECIST response is indicated for each individual and bars are colored to indicate treatment allocation (green = MET4, white = control). Dashed lines indicate +20% or −30% growth for panels A-C. (D) Best RECIST response by tumor type and treatment allocation is indicated.

CR, complete response; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; GU, genitourinary cancer; HNSCC, head and neck squamous cell carcinoma; MET4, Microbial Ecosystem Therapeutic 4; PD, progressive disease; PD-1, programmed cell death protein 1; PR, partial response; SD, stable disease.

diversity (Figure 3E) or richness (observed taxa, Figure 3F) at any timepoint. Diversity indices were not different between rarefied and non-rarefied analyses (rarefied analysis presented). In an exploratory analysis, a greater number of MET4 taxa comprised >0.01 relative abundance in the MET4-treated group than in the control group at T1 (6.7 ± 2.8 versus 4.6 ± 1.9, $P = 0.035$, Supplementary Figure S2A, available at https://doi.org/10.1016/j.annonc.2023.02.011), and the number of MET4 taxa comprising at least 0.01 of the bacterial community following MET4 exposure was greater in MET4 recipients than in controls at T2 (6.7 ± 2.0 versus 4.4 ± 2.4, $P = 0.025$, Supplementary Figure S2B, available at https://doi.org/10.1016/j.annonc.2023.02.011). Fewer patients were assessable for these ecological measures at T3 and T4 and differences observed were not statistically significant.

Post-treatment changes in MET4 taxon relative abundance varied significantly by individual and taxon (Figures 4 and 5, Supplementary Figure S3, available at https://doi.org/10.1016/j.annonc.2023.02.011). In cohort B, 8 of 17 (47%) MET4 recipients had statistically significant increases in MET4 taxa in at least one post-treatment sample (defined as a one-sample *t*-test $P < 0.05$ compared to no change), while 3 (17.6%) had decreases in at least

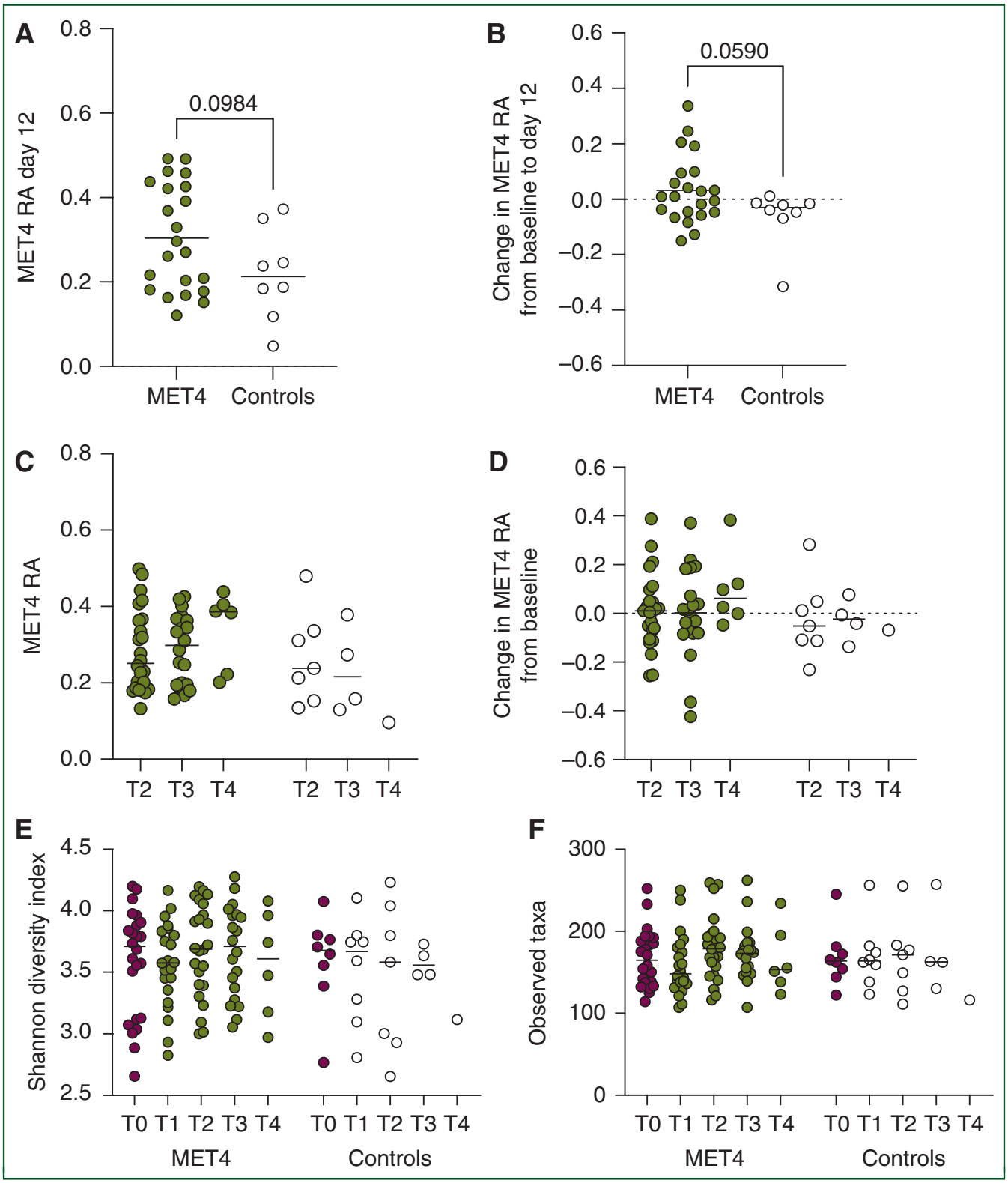


Figure 3. Ecological primary and secondary endpoints. Stool samples were collected at 3-4 weeks post-ICI/pre-MET4 (T0) and at four prespecified timepoints (day 12 post-MET4/T1, week 3-4 post-MET4/T2, week 24 post-MET4/T3 and at the end of therapy or 1 year/T4) after randomization to receive MET4 or standard-of-care ICI. 16S rRNA gene sequencing was used to determine: (A) cumulative RA of MET4 taxa and (B) change in cumulative MET4 RA. (C, D) Ecological outcomes at subsequent timepoints are shown. E, F The Shannon diversity index and observed taxa are shown for all timepoints for both groups and did not differ at any timepoint.
ICI, immune checkpoint inhibitor; MET4, Microbial Ecosystem Therapeutic 4; RA, relative abundance; rRNA, ribosomal RNA.

one sample, compared to none with increases and 3 (50%) with decreases in the controls. For cohort A, one patient had an increase and one patient had a decrease in MET4 taxa, and for cohort C, one MET4 recipient and two controls had a decrease. Notably, several individuals had >10-fold increases in multiple taxa, with increases in as many

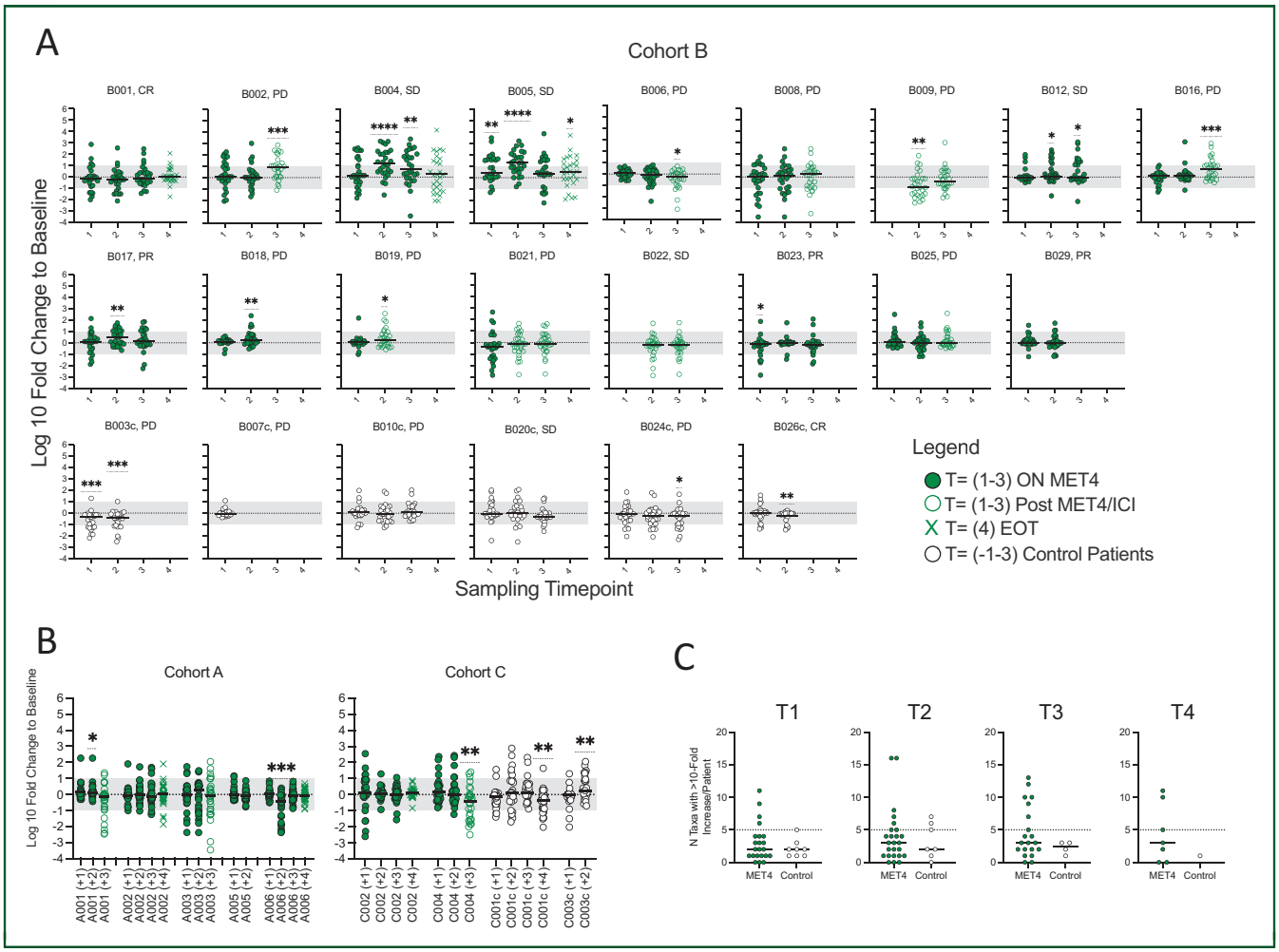


Figure 4. Changes in MET4 relative abundance by individual. Log fold change in relative abundance of MET4 taxa between baseline and subsequent timepoints is plotted for individual patients in groups B (A) and A/C (B). Each point represents the log fold change of a single MET4 taxon. One-sample *t*-tests were carried out to test whether taxonomic fold change was non-zero. Markers for the MET4 group are marked by whether the individual was on MET4 (filled circles), had discontinued MET4 >1 week before providing a stool sample (green empty circles) or was at the prespecified end of therapy (X). Controls are empty circles. (C) The number of taxa/patient with >10-fold increase in relative abundance compared to baseline at each timepoint. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, one-sample *t*-test.
CR, complete response; MET4, Microbial Ecosystem Therapeutic 4; PD, progressive disease; PR, partial response; SD, stable disease.

as 16 taxa seen at some timepoints. For example, patients B004 and B005 had >10-fold increases in the relative abundance of 9 and 11 MET4 taxa at T1, respectively, and 16 taxa each at T2. Changes in relative abundance varied by MET4 taxon, with significant increases in *Bifidobacterium*, *Enterococcus*, *Eubacterium eligens*, *Phascolarctobacterium succinatutens*, *Collinsella aerofaciens* and *Ruminococcus torques* in MET4 recipients after treatment, with a general decrease in MET4 taxa observed in controls (Figure 5A and B, Supplementary Figure S3, available at https://doi.org/10.1016/j.annonc.2023.02.011). Although inter-individual differences in ecological responses were evident, there were no generalizable differences in 16S community composition between MET4 recipients and controls, or pre-/post-MET4 treatment timepoints, and the strongest predictor of microbial community composition was trial participant (Supplementary Figures S4 and S5, available at https://doi.org/10.1016/j.annonc.2023.02.011).

Collectively, these data indicate that MET4 administration achieves measurable increases in MET4 taxa in a subset of MET4 recipients, but not controls, including increases in >5 MET4 taxa in 35% of MET4 recipients and significant increases in multiple MET4 genera, including several previously implicated in ICI responsiveness.

Baseline ecological and post-treatment metabolomic differences in MET4 ecological responders/non-responders

Amongst MET4 recipients, variable ecological responses were observed. We thus stratified MET4 recipients into those with and without an ecological response, defined as an increase of at least five MET4 taxa by at least 10-fold (a level which was associated with greater than median post-treatment MET4 relative abundance). We first assessed pre-MET4 treatment samples for predictors of ecological response/non-response. We did not observe statistically significant differences in baseline stool microbial diversity (Shannon diversity index, observed taxa, inverse Simpson) between EcoRs and EcoNRs (Supplementary Figure S6A-C, available at https://doi.org/10.1016/j.annonc.2023.02.011).

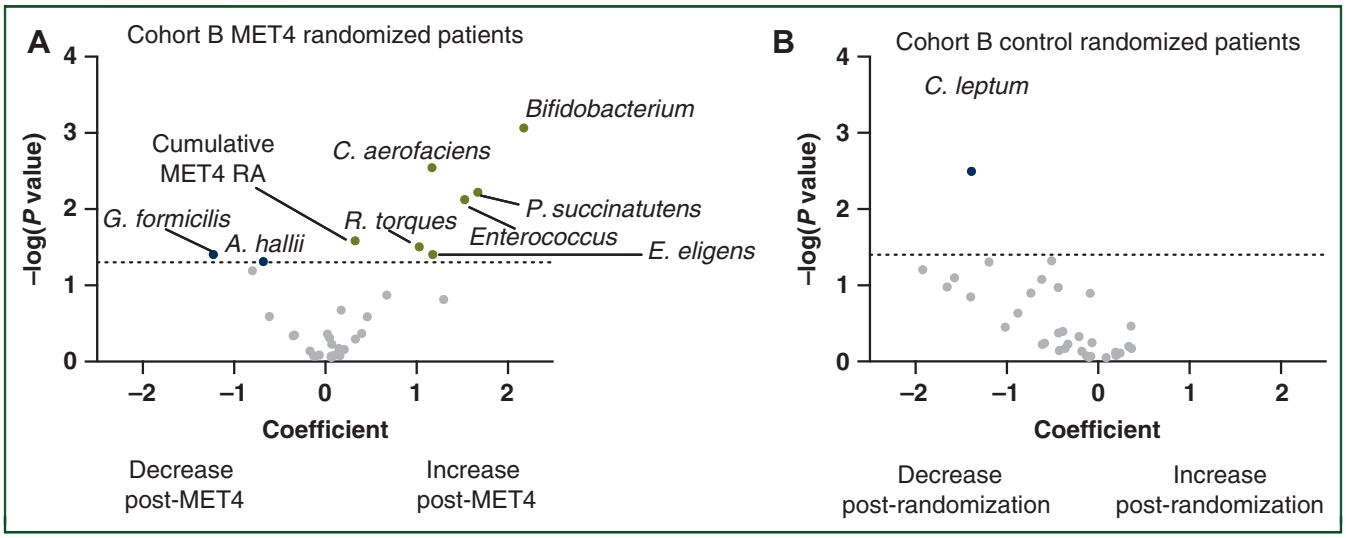


Figure 5. Volcano plots depicting differentially abundant MET4 taxa post-randomization compared to samples collected before randomization. (A) Increase (green) and decrease (blue) in MET4 taxa post-MET4 initiation. (B) Decrease in MET4 taxa post-alpha diversity randomization to the control arm. Grey dots include features that were not significantly different. MET4 taxa and alpha diversity metrics were log transformed and analyzed using MaAsLin2. Fixed effects included MET4 versus control randomization and pre- versus post-treatment; patient was set as a random effect to account for repeated measures. Benjamini—Hochberg correction was used to adjust for multiple tests with an FDR threshold of 0.25.
FDR, false discovery rate; MET4, Microbial Ecosystem Therapeutic 4.

Twenty-one of 28 MET4 taxa trended toward lower abundance at pre-MET4 initiation timepoints (T −1, T0) in EcoRs than in EcoNRs, 3 of which were significant before correction for multiple comparisons (Supplementary Figure S6D, available at https://doi.org/10.1016/j.annonc.2023.02.011). While not definitive in this limited dataset, pre-treatment colonization with endogenous MET4 taxa may inhibit MET4-induced ecological responses, or conversely that low MET4 species relative abundance and/or alpha diversity allows MET4 engraftment.

Recently, ICI responsiveness after FMT in patients with refractory melanoma was correlated with changes in microbial metabolites including increased transformation of primary to secondary BAs.[5] We therefore assessed the subset of cohort B patients in whom plasma samples were available at T0 ($n = 25$ samples) and T1 ($n = 25$ samples) and T2 ($n = 18$ samples) by targeted metabolomics. No significant differences were observed in plasma SCFAs and BAs between MET4 recipients and controls, or between MET4 recipients who had an ecological response and those without (Supplementary Figure S7, available at https://doi.org/10.1016/j.annonc.2023.02.011). There were no differences between timepoints or treatment groups in plasma BAs; however, three primary BAs decreased in individuals who had ecological engraftment (Supplementary Figure S8, available at https://doi.org/10.1016/j.annonc.2023.02.011), suggesting that engraftment may be associated with measurable changes in metabolites in plasma that have previously been associated with ICI response after FMT.[5] Stool SCFA and BA levels were similarly assessed. No significant differences in stool SCFA were observed across timepoints between treatment groups, or were there differences in change in SCFA levels between EcoRs, EcoNRs and controls (Supplementary Figure S9, available at https://doi.org/10.1016/j.annonc.2023.02.011). Similar to plasma, stool primary BAs did not differ between treatment groups across timepoints (Supplementary Figure S10A, available at https://doi.org/10.1016/j.annonc.2023.02.011), but decreases in primary BAs were noted in EcoRs, but not in EcoNRs or controls after treatment (Supplementary Figure S10B, available at https://doi.org/10.1016/j.annonc.2023.02.011), indicating that MET4-associated ecological response is associated with metabolic changes in both plasma and stool.

DISCUSSION

In this first-in-human trial of a cultivated microbial consortium administered as a co-therapy for ICI, we found that MET4 was well tolerated, with no high-grade AEs or worsening of ICI-associated irAEs, and that MET4 administration was associated with significant increases in therapeutic taxa in a subset of individuals. This engraftment was associated with peripheral metabolome changes recently associated with response to ICI after FMT.[5]

Interest in FMT as a microbiome-remediating strategy for both infectious and non-infectious diseases has increased significantly since FMT by duodenal infusion was shown to be effective for the treatment of recurrent *C. difficile* infection in a human interventional trial.[11] Multiple studies of FMT as a co-therapy for ICI are registered on ClinicalTrials.gov, including several phase II or phase I-II trials.[5,12-17] However, because safety, reproducibility and barriers to production at scale significantly limit the use of FMT, alternative strategies are needed. While single- or limited-strain probiotics are an alternative microbiome-targeting strategy, they have important caveats as a co-therapy to ICI. Firstly, probiotic effects on the composition of the microbiome do not reproduce the ecological effects of FMT in individuals with low microbial diversity and are

associated with decreased gut microbiome diversity compared to no treatment or FMT.[18] Secondly, in ICI recipients, limited complexity probiotic use may be associated with decreased ICI responsiveness, whereas dietary fiber, which promotes a complex and diverse microbiome, is associated with ICI response.[19] Thirdly, in cross-cohort analyses, no single species has emerged as uniformly ICI response-associated.[20] An important caveat to this observation is that the studies included were relatively small and are thus not definitive; however, it is also possible that 'narrow-spectrum' microbial therapies may not adequately reproduce the ecological and functional complexity of ICI response-associated microbiomes. Alternatives to FMT as an ICI co-therapy will ideally promote ecologically complex, multi-species responses in the recipient and be safe, tolerable and ecologically and physiologically significant in ICI recipients. There are a total of three clinical trials registered for evaluating microbial consortia as an ICI co-therapy,[21-23] and to our knowledge, our study is the first report of a microbial consortium used in combination with ICI in advanced cancer patients. A randomized phase I study of CBM588, a *Clostridium butyricum*-containing probiotic designed to promote Bifidobacteria,[24] in combination with anti-PD-1 and anti-CTLA-4 antibodies in ICI-naïve metastatic renal cell carcinoma patients, failed to meet its primary endpoint of a change in *Bifidobacterium* spp. at 12 weeks. Interestingly, statistically significant longer progression-free survival in the investigational treatment arm as compared to the control arm was observed in this small study. However, an imbalance in patients with poor international metastatic database consortium risk score was noted between the two arms. In contrast to this report, we evaluated a novel microbial consortium in which microbial species function in an ecologically complex manner.

In our trial, MET4 was tolerable and delivered safely in ICI recipients regardless of tumor type, indicating that this novel therapeutic approach may be feasible broadly in ICI recipients. We observed ecological response in a proportion of MET4 recipients, which included increases in multiple taxa that have been associated with ICI responses, such as *Enterococcus*, *Bifidobacterium* and *Phascolarctobacterium*, and we also observed changes in metabolites associated with ecological response. These results indicate the viability of microbial consortia as an ICI co-therapy. However, there are several important observations and limitations of this study and our findings are exploratory in nature. We are not adequately powered to assess the reasons for variability in ecological responsiveness in this small study. Notably unlike recent trials of FMT which enrolled ICI-resistant patients,[5,6] this study mainly included ICI-naïve patients receiving immunotherapy as standard of care; therefore, a response would be expected regardless of the addition of MET4. Populations with prior non-response or recent antimicrobial exposure may demonstrate different ecological responses to MET4. The association between ecological responsiveness and clinical response could not be assessed in this early-phase trial, especially given the heterogeneity of tumor types and variability of ICI regimens in the enrolled patients. We did not collect fresh tumor biopsies in this trial, therefore unable to assess the impact of MET4 administration or ecological responsiveness on the circulating or tumor immune phenotype. Finally, our sequencing approach was not able to distinguish between endogenous and exogenous MET4 strains. In spite of these limitations, we believe that the presence of engraftment in some MET4 recipients, changes in plasma and stool metabolite concentrations associated with engraftment and safety and tolerability of the intervention justify the pursuit of a larger trial of microbial consortia in ICI recipients with solid tumors. A pan-Canadian, randomized, placebo-controlled phase II trial in PD-L1-selected patients with recurrent/metastatic squamous cell cancer receiving anti-PD-1 antibody has been endorsed by the Canadian Cancer Trial Group. This study will evaluate the efficacy of MET4 as an adjunct to ICI (https://www.ctg.queensu.ca/public/head_neck/head-neck-disease-site) with comprehensive correlative predictive and pharmacodynamic biomarker evaluation of tumor, blood and stool samples.

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DISCLOSURE

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employed by Nubiyota. EAV is co-founder and CSO of NuBiyota LLC. All other authors have declared no conflicts of interest.

DATA SHARING

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. All sequence data are availabile in GenBank/NCBI under the accession numbers PRJNA835435 and PRJNA819052.

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Hypothesis

1. Patients with Human Papillomavirus (HPV)-related locally-advanced oropharyngeal squamous cell carcinoma (LA-OPSCC) harbor specific oral and intestinal microbiome composition before definitive chemoradiotherapy.
2. Chemoradiotherapy has an impact on both oral and intestinal microbiome composition and diversity.
3. Modulation of intestinal microbiome with the oral bacterial consortia MET-4 (a mixture of bacterial species related to the immune response) is feasible and safe in the context of concurrent definitive chemoradiotherapy in patients with HPV-related LA-OPSCC.
4. MET-4 administration will lead to qualitative and quantitative changes in gut microbiome composition.
5. The engraftment of MET-4 IO-responsiveness taxa is associated with specific changes in stool and plasma metabolomes.

Objectives

1. To characterize baseline and post-treatment oral and intestinal microbial composition in saliva, tumor swabs and stool samples in a prospective cohort of patients with newly-diagnosed HPV-related LA-OPSCC treated with definitive chemoradiotherapy.
2. To evaluate the effect of chemoradiotherapy on both oral and gut microbiome composition in terms of relative abundance and diversity.
3. To investigate the feasibility and safety of MET-4 administration in the context of chemoradiotherapy in a prospective cohort of HPV-related LA-OPSCC.
4. To assess the engraftment of MET-4 associated taxa – defined as the qualitative and quantitative presence of the bacterial strains in the stools following MET-4 administration- when given in combination with definitive chemoradiotherapy.
5. To evaluate the changes in stool and plasma metabolomes resulting from the engraftment of MET-4-associated taxa.

Material, Methods and Results.

Article 1. Transitions in oral and gut microbiome of HPV+ oropharyngeal squamous cell carcinoma following definitive chemoradiotherapy (ROMA LA-OPSCC study).

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ARTICLE

Molecular Diagnostics

Transitions in oral and gut microbiome of HPV+ oropharyngeal squamous cell carcinoma following definitive chemoradiotherapy (ROMA LA-OPSCC study)

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BACKGROUND: Oral and gut microbiomes have emerged as potential biomarkers in cancer. We characterised the oral and gut microbiomes in a prospective observational cohort of HPV+ oropharyngeal squamous cell carcinoma (OPSCC) patients and evaluated the impact of chemoradiotherapy (CRT).

METHODS: Saliva, oropharyngeal swabs over the tumour site and stool were collected at baseline and post-CRT. 16S RNA and shotgun metagenomic sequencing were used to generate taxonomic profiles, including relative abundance (RA), bacterial density, α-diversity and β-diversity.

RESULTS: A total of 132 samples from 22 patients were analysed. Baseline saliva and swabs had similar taxonomic composition ($R^2 = 0.006$; $p = 0.827$). Oropharyngeal swabs and stool taxonomic composition varied significantly by stage, with increased oral RA of *Fusobacterium nucleatum* observed in stage III disease ($p < 0.05$). CRT significantly reduced the species richness and increased the RA of gut-associated taxa in oropharyngeal swabs ($p < 0.05$), while it had no effect in stool samples. These findings remained significant when adjusted by stage, smoking status and antibiotic use.

CONCLUSIONS: Baseline oral and gut microbiomes differ by stage in this HPV+ cohort. CRT caused a shift towards a gut-like microbiome composition in oropharyngeal swabs. Stage-specific features and the transitions in oral microbiome might have prognostic and therapeutic implications.

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BACKGROUND

The human microbiome has recently emerged as a promising biomarker in cancer.[1] The microbiome inhabiting the oro-gastrointestinal tract has been implicated in the carcinogenesis of many tumour types and in modulating responses to anti-cancer therapies, including immunotherapy, although the mechanisms are not yet well understood.[2–4] A few studies have shown differential oral microbial composition in the saliva of patients with oral cavity and oropharyngeal tumours when compared to healthy individuals, while specific commensals have been associated with lower risk of developing head and neck squamous cell carcinomas (HNSCC).[5–7] Oral microbiome composition seems to vary across different primary sites (e.g. oral cavity vs oropharynx) or according to stage, human papillomavirus (HPV) status and treatment received (e.g. surgery vs chemoradiotherapy (CRT)), suggesting a role as a tumour-specific biomarker in this disease, with potential impact on treatment efficacy and toxicity.[8–10] However, the evaluation of the oral microbiome in HNSCC has thus far been limited to retrospective and heterogeneous cohorts of patients, while the gut microbiome is yet to be investigated.

Among HNSCC, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) has dramatically increased over the past decade, with HPV-related disease being most prevalent.[11,12] HPV-positive (HPV+) OPSCC are a biologically distinct disease with increased treatment responsiveness and survival when compared to HPV-negative tumours.[13] As such, multiple studies are evaluating de-escalation strategies in the locoregionally advanced (LA) setting to reduce treatment toxicity without compromising

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survival.[14] However, HPV+ tumours are heterogeneous and not all have a favourable prognosis.[15] Beyond clinical and pathological factors such as smoking history, tumour, node, metastasis (TNM) staging and HPV status, there remains an unmet need for new biomarkers that provide accurate risk stratification of this patient population. In this regard, HPV+ LA-OPSCC represents a unique setting to evaluate and compare both tumour-associated and gut microbiomes and their potential effect on treatment.

ROMA LA-OPSCC is the first study to prospectively characterise both oral and gut microbiomes and to evaluate the impact of definitive CRT on their composition in a homogeneous cohort of newly diagnosed HPV+ LA-OPSCC.

METHODS

Patient population and study design

ROMA LA-OPSCC (NCT03759730) is a single-centre, non-interventional, investigator-initiated feasibility study designed to evaluate the oral and intestinal microbiome in a prospective cohort of patients with HPV+ LA-OPSCC treated with definitive CRT. Patients with previously untreated histologically proven OPSCC (tonsil, base of tongue, soft palate) candidates for definitive concurrent CRT with single-agent cisplatin (CDDP) as per standard of care were eligible. HPV status was determined by p16 immunohistochemical staining and classified as positive if nuclear and cytoplasmic staining in ≥70% tumour cells. In situ hybridisation to confirm the presence of high-risk HPV DNA was performed in equivocal cases. All patients were staged and treated according to eighth edition TNM staging criteria. Treatment and follow-up assessments were conducted according to institutional protocol (Supplement). Saliva, oropharyngeal swabs over the tumour site and stools samples were collected before treatment (up to 3 weeks prior to the start of radiotherapy) and at completion of CRT (up to 3 weeks following last day of radiotherapy; Supplementary Fig. 1). Patients were evaluable for analysis if samples were provided at least at one time point. The study was approved by the institutional research ethics board. All patients provided written, signed, informed consent to participate.

Treatment and follow-up

All patients received intensity-modulated radiotherapy to a gross tumour dose of 70 Gy in 35 fractions over 7 weeks (2 Gy/fraction). Concurrent CDDP (three-weekly at 100 mg/m^2 on RT days 1, 22 and 43 or weekly at 40 mg/m^2 for 7 weeks) was delivered according to institutional protocol. The choice of a three-weekly versus weekly schedule was based on patient's Eastern Cooperative Oncology Group Performance scale and comorbidities as assessed by medical oncologist. All patients had a prophylactic gastrostomy tube placed within 3–4 weeks from the start of radiation as per institutional standard practice. Follow-up after treatment completion was conducted according to institutional protocol. Local and regional recurrences were confirmed histologically, while distant metastases were diagnosed by unequivocal clinical/radiologic evidence +/− histologic confirmation. Clinical data were abstracted prospectively (M.O.) for all patients enrolled in the study.

Sample collection and microbiome analysis

Saliva, oropharyngeal swab over the tumour site and stool samples were collected using the ZymoBIOMICS DNA/RNA Mini Prep™ kits (Zymo Research, Irvine, CA). Sampling and storage protocol are available as Supplementary Data (Laboratory Manual). Processing and analysis of the samples was conducted at the Centre for Genome Evolution and Function (CAGEF) of the University of Toronto. DNA was extracted using the ZymoBIOMICS DNA Micro Kit™. 16S rRNA gene sequencing (Supplement) was performed on saliva ($n = 46$), oropharyngeal swabs ($n = 46$) and stool samples ($n = 46$). Briefly, the V4 hypervariable region of the 16S rRNA gene is amplified using an universal forward sequencing primer and a uniquely barcoded reverse sequencing primer to allow for multiplexing.[16] Amplicon sequencing was performed on an Illumina MiSeq platform (Illumina, CA, USA) with V2 chemistry as described in Schneeberger et al.[17]. Taxonomic profiling of 16S data sets was performed using the UNOISE pipeline.[18] Shotgun metagenomics sequencing was only performed on oropharyngeal swabs ($n = 46$) and stool samples ($n = 46$). Libraries were constructed using the Illumina Nextera Flex kits (Illumina, USA) using 150 ng DNA as input. A total of 1.94 Billion reads were generated on an Illumina NovaSeq 6000 platform (Illumina, USA) using a SP flow cell and reagents according to the manufacturer's protocol at the Princess Margaret Genomics Centre. A median of 2.3E + 07 [1.35E + 07–4.01E + 07] reads for stool samples and of 9.16E + 05 [2.17E + 05–3.09E + 07] reads for oropharyngeal samples were remaining after host read removal with Kneaddata v. 0.7.2 (https://bitbucket.org/biobakery/biobakery/wiki/kneaddata). Taxonomic profiles resulting from shotgun datasets were generated using Metaphlan2 with the Chocophlan database v. 293.[19,20] Alpha diversity and beta diversity were measured using the Phyloseq package (ref. [20]; v. 3.9) and VEGAN v. 2.5.5[21] in R v. 3.5.3.[22]

Statistical analysis

ROMA LA-OPSCC is a signal-finding study. Descriptive statistics were used to summarise clinical and microbiome characteristics. Mixed model regression was conducted to explore the potential demographic and clinical factors that are related to the microbial change during CRT. For microbiome analyses, summary statistics were described including within-patient community composition (taxonomic relative abundance), alpha diversity (compositional diversity within-sample) using Shannon index (SDI; a composite metric of both richness and evenness) and Berger–Parker index (BP; an indicator of dominance in the community), as well as beta-diversity (inter-sample similarity) of baseline and end of treatment samples. Alpha diversity measures were compared between groups using Mann–Whitney (MW) tests. LEFSE was used to measure the differences in relative abundances (non-parametric Kruskal–Wallis tests) and the effect size (linear discriminant analysis) between groups. Beta diversity was measured using the Bray–Curtis dissimilarity index and group comparisons were conducted using permutational multivariate analysis of variance (PERMANOVA). Interaction between treatment effect (changes in composition pre- and post-CRT) and other variables including use of antibiotics, G-tube dependency, grade of mucositis, smoking status, tumour location, stage and T staging were measured using PERMANOVA. Assuming a significance level for alpha of 0.01 to adjust for multiple comparisons of key taxa, alpha diversity, and beta diversity, our study with 22 patients' microbiome samples had at least 85% power to identify significant differences between pre- and post-CRT, given an effect size of 0.7 standard deviation (SD) of the paired mean difference. The power analysis is based on two-sided paired *t* tests.

RESULTS

Clinical characteristics and outcome

From January 2018 to November 2018, 26 patients with newly diagnosed LA-OPSCC candidates for CRT were enrolled in the study, of which 22 were included in this analysis. Four were excluded for reasons outlined in the Consort diagram (Supplementary Fig. 2). Patient characteristics are summarised in Supplementary Table 1. Most patients were male and smokers (current or former) with ≥10 pack-year smoking history. Thirty-six percent of patients had stage III disease at presentation with tonsil being the most common primary site. Eleven patients received antibiotics up to 1 month prior to and/or during CRT. At the time of data cut-off, with a median follow-up of 90 weeks (20–115), all

patients were alive and 21/22 were disease free. One patient (R05) developed locoregional and distant recurrence.

Description of baseline oral and stool microbiome in HPV+ LA-OPSCC

A total of 132 samples collected from the 22 evaluable patients (100% compliance in sample acquisition) were analysed. Taxonomic composition of oropharyngeal swabs and saliva samples by 16S rRNA gene sequencing were similar ($R^2 = 0.06$; $p = 0.827$; Supplementary Fig. 3), thus shotgun metagenomic sequencing was only conducted in oropharyngeal swabs and stool. All subsequent results are based on shotgun metagenomic sequencing analyses. Taxonomic composition differed by sampling site (oropharyngeal swabs vs stool samples: $R^2 = 0.276$; $p = 0.001$; Fig. 1a, b). Oral communities comprised mostly oropharyngeal anaerobes and facultative anaerobes, including *Prevotella*, *Veillonella*, *Streptococcus* and *Actinomyces* species while stool communities were composed mainly of obligate anaerobic *Bacteroides* species. The number of species was higher in the stool vs oral communities ($p < 0.0001$) but they had overall similar diversity ($SDI_{mean} = 3.3$ for stool and 3.12 for oropharyngeal samples; $BP_{mean} = 0.19$ for stool and 0.2 for oropharyngeal samples; Fig. 1c). Four patients (R05, R17, R23 and R26) had a high proportion (>10% of the community) of *Bacteroides* species in their oropharyngeal swabs more typical of the lower intestinal tract.

Differential baseline oral and stool microbiome composition by stage

Taxonomic composition of oropharyngeal swabs significantly differed across stage III vs stage I–II patients ($p < 0.05$): four genera were enriched in patients with stage III, including *Fusobacterium* (*Fusobacterium nucleatum*), *Gemella* (*Gemella morbillorum* and *Gemella haemolysans*), *Leptotrichia* (*Leptotrichia hofstadii*) and *Selenomonas* (*Selenomonas sputigena* and *Selenomonas infelix*) (Fig. 2a). Taxonomic composition of stool samples also differed in stage III vs stage I–II disease, with significant enrichment of two phyla, *Actinobacteria* and *Proteobacteria*, and 18 species ($p < 0.05$; Fig. 2b).

In the univariate analysis, no effect on baseline oropharyngeal swab microbiome composition was seen by smoking or primary tumour location, although a trend was observed by T staging ($p = 0.06$; Supplementary Table 2).

Impact of CRT on the oral and stool microbiome

Oral microbiome. We compared the composition of oropharyngeal swabs pre- and post-CRT (Fig. 3). We observed the formation of three distinct clusters based on collection time point baseline vs post-CRT (Fig. 3a): cluster 1 (15 baseline vs 1 post-CRT samples) was characterised by high relative abundance of species from the *Veillonella*, *Prevotella* and *Streptococcus* genera; cluster 2 (4 baseline vs 11 post-CRT samples) was characterised by high abundances of *Streptococcus* species, *Prevotella melaninogenica*, *Neisseria flavescens* and *Rothia mucilaginosa*, among others; and cluster 3 (3 baseline vs 10 post-CRT samples) was characterised by high abundances of species from the *Bacteroides*, *Faecalibacterium*, *Prevotella* (*Prevotella copri*), *Collinsella*, *Alistipes* and *Parabacteroides* genera. Overall, the number of species was significantly reduced in post-CRT oropharyngeal swabs (MW; $p = 0.006$). Alpha diversity did not change post-CRT ($SDI_{mean} = 3.12$ at baseline and 3.09 at the end of treatment; $BP_{mean} = 0.2$ at baseline and 0.2 at the end of treatment; MW; $p_{SDI} = 0.716$; $p_{BP} = 0.944$) nor did bacterial density (8.8E + 09 16S copies/ml at baseline and 2.6E + 09 16S copies/ml at the end of treatment; $p = 0.15$) (Supplementary Fig. 4). Intra-patient changes in community composition post-CRT are summarised in Fig. 3b. Most patients (65%) were classified in cluster 1 at baseline while only 1 patient (4.5%) was classified in cluster 1 post-CRT. Out of the 15 patients in cluster 1 at baseline, 8 transitioned to cluster 2 and 6 to cluster 3 post-CRT. None of the patients who grouped in cluster 2 and 3 at baseline shifted to cluster 1 after CRT and remained within the cluster 2 or 3.

Clinical characteristics associated with cluster subgroups are summarised in Table 1. Out of the 15 patients grouping in cluster 1 at baseline, the majority were former/non-smokers (93%), with tonsillar primary (67%) and stage I–II disease (67%). Baseline oral composition from 3 out of the 4 current smokers of the cohort belonged to cluster 2 or 3. No clear pattern was seen between cluster transitions post-CRT by TNM, stage, smoking status, use of antibiotics, grade of mucositis or gastrostomy tube dependency post-CRT. One of the two patients (patient R05) with cluster 3-type oral microbial composition at baseline and post-CRT experienced biopsy-proven locoregionally and distant recurrence.

Overall, the compositional changes between baseline and post-CRT consisted of a shift towards gastrointestinal tract-like communities (Fig. 4). Oropharyngeal swabs post-CRT clustered closer to stool samples, with significant changes in taxa composition when compared to baseline ($R^2 = 0.1$; $p = 0.001$). The dissimilarity observed between oropharyngeal swabs and stool samples was reduced in post-CRT samples (PERMANOVA; $R^2 = 0.115$; $p = 0.001$) compared to baseline samples (PERMANOVA; $R^2 = 0.203$; $p = 0.001$). Functional analyses associated with these taxonomic findings were attempted but could not be performed due to insufficient sequencing depth (Supplementary Fig. 5).

Stool microbiome. No clustering was observed in stool samples by collection time point (baseline vs post-CRT); similar taxa composition and alpha diversity was observed post-CRT (Supplementary Fig. 6). No differences in taxonomic composition were observed in post-treatment samples based on the use of antibiotics (Supplementary Table 3 and Supplementary Fig. 7).

The impact of CRT (defined as collection time point: baseline and post-CRT) on the oral microbiome remained significant when adjusting by potential confounding factors, including smoking status, TNM, stage subgroups, maximum grade of mucositis, gastrostomy tube dependency 3–4 weeks post-CRT and use of antibiotics (Supplementary Table 4).

DISCUSSION

ROMA LA-OPSCC is the first study to prospectively characterise both oral and gut microbiomes in HPV+ OPSCC patients treated with definitive CRT. We found that both oral and stool community composition differed by disease stage at baseline and that the oral but not stool microbiome composition changed after CRT. The shift in oropharyngeal taxonomic composition after treatment was largely driven by an increase in the relative abundance of gut-associated obligate anaerobes. The results of this study provide a step forward in the understanding of both microbiomes in this disease and may be used as a benchmark as new treatments are being investigated in this patient population.

The composition of the oral microbiome in our cohort was comparable to that of other retrospective cohorts involving patients with oral cavity and oropharyngeal tumours.[8,23] Guerrero-Preston et al. reported differential taxonomic composition in HPV+ OPSCC when compared to HPV-negative OPSCC and oral cavity cancer, with higher prevalence of *Veillonella*, *Prevotella*, *Streptococcus* and *Gemella* genera.[8,9] In our cohort involving HPV+ disease exclusively, we did observe a similar taxonomic composition at the genus level. Our shotgun metagenomics analysis revealed differential oral microbial composition across stages, and patients with stage III had significantly higher relative abundance of *F. nucleatum* species. *F. nucleatum* had been previously described in heterogeneous cohorts involving HNSCC patients treated with surgery and/or radiation, but it has also been recently associated with advanced disease, chemotherapy resistance and adverse prognosis in other tumour

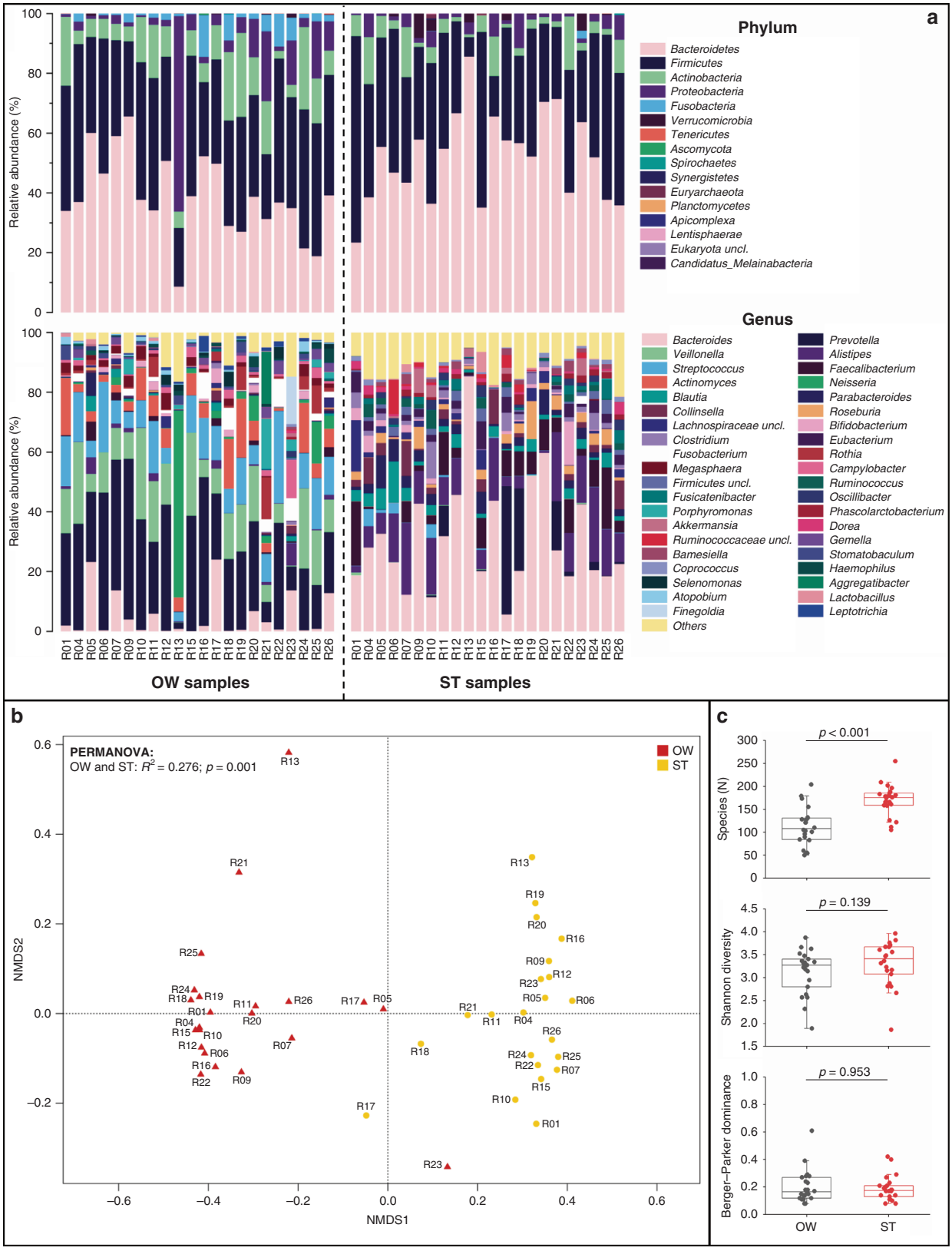

Fig. 1 Taxonomic composition differed by sampling site (oropharyngeal swabs vs stool samples). a Community composition of oral microbiome at the phylum and genus levels. Species-level composition is summarised in Supplementary Table 4. **b** Non-metric multidimensional scaling plot comparing taxonomic composition of sample types at baseline. **c** Alpha diversity indices in stool and oral communities, including identified species (upper panel), Shannon diversity (central panel), and Berger–Parker dominance (lower panel). OW oropharyngeal swabs over the tumour site, ST stools.

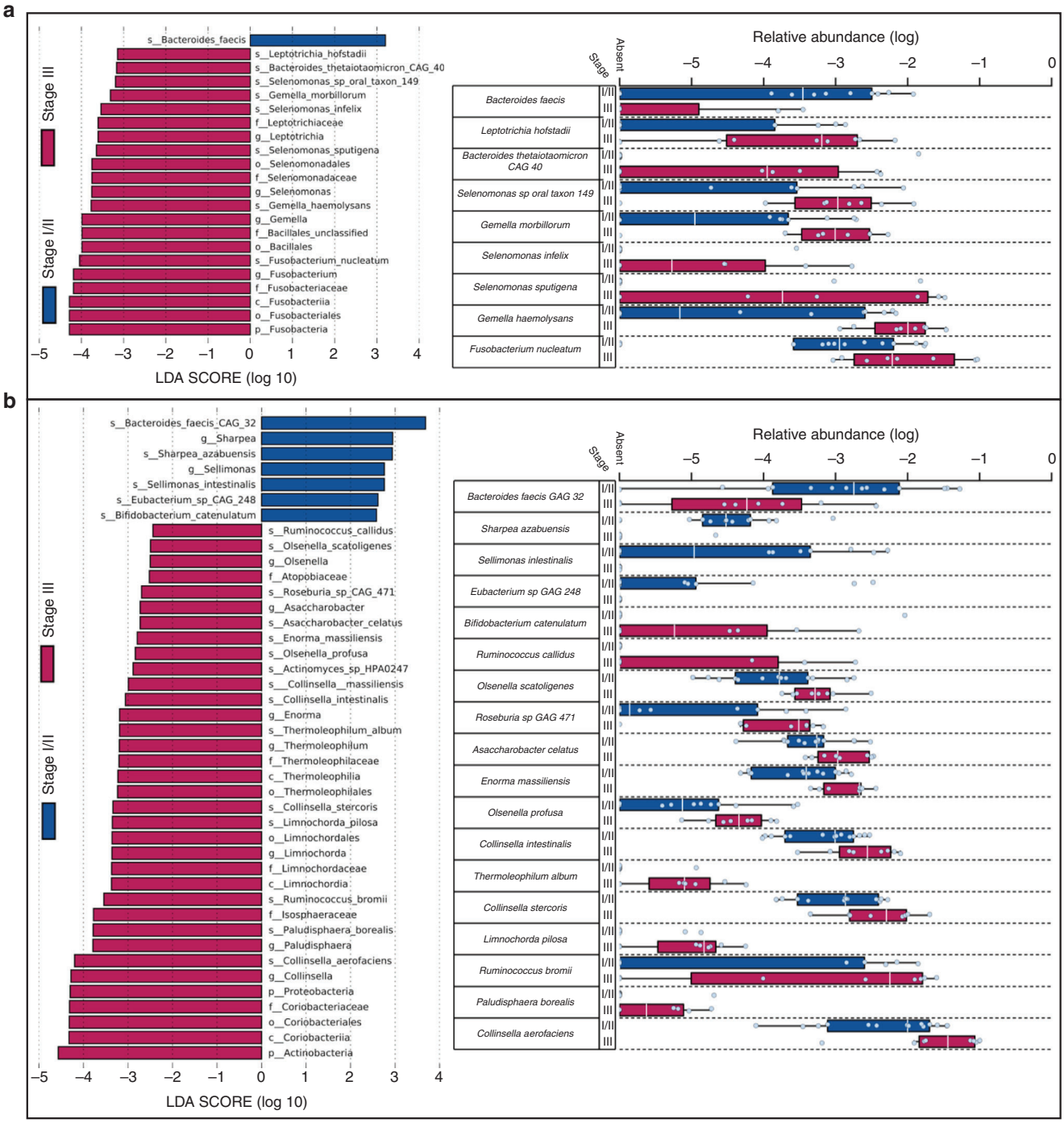

Fig. 2 Compositional differences in the oral and intestinal communities differed by disease stage. a Compositional differences in the oral communities at baseline. The left panel indicates taxonomic features (at all taxonomic levels) different in abundance at early (stage I/II) and advanced (stage III) disease stage (LDA > 2.0; $p < 0.05$). The right panel shows the difference in normalised relative abundance of enriched/depleted species. **b** Compositional differences in the gut communities. The left panel highlights all differentially abundant taxonomic features between early and advanced disease stage (LDA > 2.0; $p < 0.05$). The right panel shows the difference in normalised relative abundance of identified species. The LDA score indicates the effect size of the differences observed between groups.

types, such as oesophageal carcinoma.[9,24–26] In our cohort, the stage differences in oral composition seemed to be associated with larger primary tumours. Patients with stage III HPV+ OPSCC are known to be at higher risk of recurrence despite definitive concurrent chemoradiation and new treatment intensification approaches including immunotherapy that are being explored in this setting (e.g. NCT03040999).[27] It remains to be tested whether these findings have prognostic implications and therefore could be used for risk stratification in this patient population. Interestingly, while smoking history seems to have a role as prognostic biomarker for HPV+ disease and has also been highlighted to correlate with oral dysbiosis, we did not observe differences in oral microbiome composition according to smoking status in our overall cohort or by stage.[28–30]

We evaluated the changes post-CRT on both oral and stool microbiomes. Two studies involving patients with HNSCC and

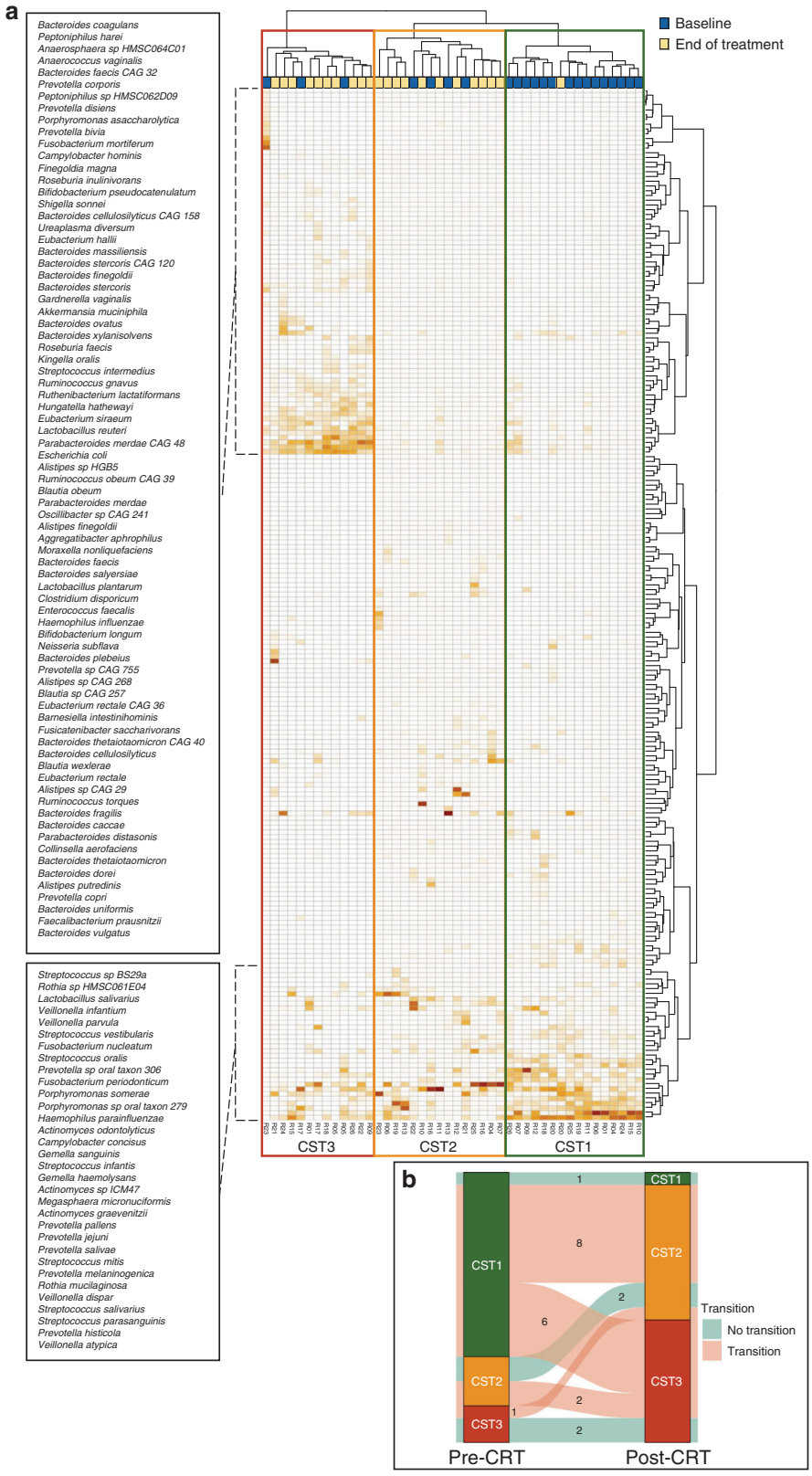

Fig. 3 Compositional differences in oral communities based on collection time point (baseline vs post-CRT). a Taxonomic composition of pre-/post-treatment oral microbiome clustered using Bray–Curtis dissimilarity (columns) and Ward's clustering method (rows). **b** Community transitions observed in oropharyngeal swabs after CRT (paired samples). The number of patients which transition from one community to another after CRT is indicated with each arrow. CRT chemoradiotherapy.

Table 1. Patient baseline and post-CRT community state types matched with clinical characteristics.

Patient ID	BSL cluster	EOT cluster	Tumour location	TNM	Stage	Smoking status	Antibiotic use	Grade of mucositis	Gastrostomy dependancy[a]
R01	1	3	Tonsil	T3N2b	II	Current	No	3	Yes
R04	1	2	Base of tongue	T1N3	III	Never	Yes	3	UK
R06	1	2	Tonsil	T3N2	II	Never	No	2	Yes
R07	1	2	Tonsil	T2N3	III	Former	Yes	2	Yes
R09	1	3	Tonsil	T2N1	I	Never	Yes	2	Yes
R10	1	2	Tonsil	T4N1	III	Never	No	2	Yes
R11	1	2	Tonsil	T3N1	II	Former	Yes	3	Yes
R12	1	2	Base of tongue	T2N1	I	Former	No	2	Yes
R15	1	3	Base of tongue	T2N1	I	Former	Yes	3	No
R18	1	3	Base of tongue	T4N1	III	Former	Yes	2	Yes
R19	1	2	Tonsil	T2N1	I	Former	Yes	1	No
R20	1	1	Soft palate	T1N1	I	Never	Yes	1	No
R24	1	3	Tonsil	T3N1	II	Never	No	3	Yes
R25	1	2	Base of tongue	T1N1	I	Never	No	1	No
R13	2	2	Base of tongue	T3N1	II	Never	Yes	2	No
R16	2	2	Tonsil	T4N0	III	Current	No	2	Yes
R21	2	3	Tonsil	T4N0	III	Current	No	2	Yes
R22	2	3	Soft palate	T4N1	III	Former	No	3	Yes
R05	3	3	Tonsil	T1N2c	II	Current	Yes	2	Yes
R17	3	3	Tonsil	T3N0	II	Former	Yes	2	Yes
R23	3	2	Base of tongue	T1N2	II	Former	No	2	No

Patient R05 (in bold) is the only patient in the cohort who experience disease recurrence.
BSL baseline, *EOT* end of treatment.
[a]G-tube dependency at the time of collection of EOT samples, up to 3 weeks from the last day of RT.

nasopharyngeal carcinoma have described changes in the oral microbiota following radiation and an increase in opportunistic pathogens.[31,32] Our analysis revealed a significant and consistent impact of CRT in the overall oral communities among the cohort, with increases in the prevalence and relative abundance of obligate anaerobes (e.g. *Bacteroides* species). The cause of these shifts is unclear but may be due to treatment-induced tissue necrosis or other changes in the tumour-adjacent mucosa, direct effects of CRT on the microbes themselves or treatment-associated immune or metabolic changes in the local tissues affecting microbial ecology. The potential biological and/or clinical impact of baseline and post-treatment composition or shifts after CRT remains unknown and long-term follow-up is required. Of note, one of the two patients harbouring a "gut-like" oropharyngeal taxa both at baseline and post-CRT experienced disease recurrence about a year after treatment completion.

We did not observe any significant shift in the gut microbiome composition after CRT in our cohort. While radiation is a local therapy and thus it is not expected to specifically alter the gut microbiome, cytotoxic chemotherapeutic agents including cisplatin are known to induce damage of the intestinal mucosa and disrupt the microbiome, leading to increased risk of infections.[33] The heterogeneity of gut microbiome composition at baseline and the limited number of patients might have limited the detection of differences due to CRT or antibiotic use. Although there were intra-patient changes in gut microbiome composition in our study, these changes were patient specific and no common pattern was observed in the overall cohort.

There were no differences in the overall taxa composition between saliva and oropharyngeal swabs taken from the tumour site. This is particularly relevant as this patient population is characterised by radiation-induced xerostomia,[34] and thus the swab could substitute the collection of saliva, the collection of which can be a challenge following completion of CRT in this patient population. Zhang et al. reported differential taxa composition between saliva and tumour tissue from patients with oral cavity tumours, with significantly higher levels of *F. nucleatum* and *Acinetobacter* found in the tumour.[25] Whether microbiome data that are obtained from oropharyngeal swabs differ from those from tumour tissue was not assessable in our study.

The limitations of our study include: inability to account for all patient factors that may influence oral and stool microbial community composition, such as dietary habits and dental hygiene; short median follow-up for HPV+ OPSCC disease limiting the evaluation of the prognostic impact of microbiome signatures; lack of further sampling beyond 4 weeks from CRT limiting the evaluation of long-term oral and stool microbiome alterations post-CRT[31,35]; small number of patients involved, which prevents statistical power for specific subgroup analysis. We used both 16S rRNA and shotgun sequencing techniques for two reasons. We first wanted to assess the level of agreement between different samples types retrieved in the same body compartment (saliva vs oropharyngeal swabs). For this exploratory analysis, 16S sequencing is sufficiently sensitive to compare the overall composition between sample types with a relatively low cost. Based on the high agreement between both sample types, we then selected oropharyngeal swabs for shotgun sequencing, as it has higher taxonomic resolution to observe CRT-mediated changes at the species level. This combination of approaches allowed us to gain the greatest amount of high-resolution microbiome compositional data at the lowest cost. A 'shallow' shotgun approach was used to characterise the taxonomic composition in the different sample types, which was sufficient for the detection of species above relative abundance of 0.05% but it did not allow us to conduct functional analyses.[36]

This pilot study shows that prospective characterisation of both oral and stool microbiome is feasible in this patient population,

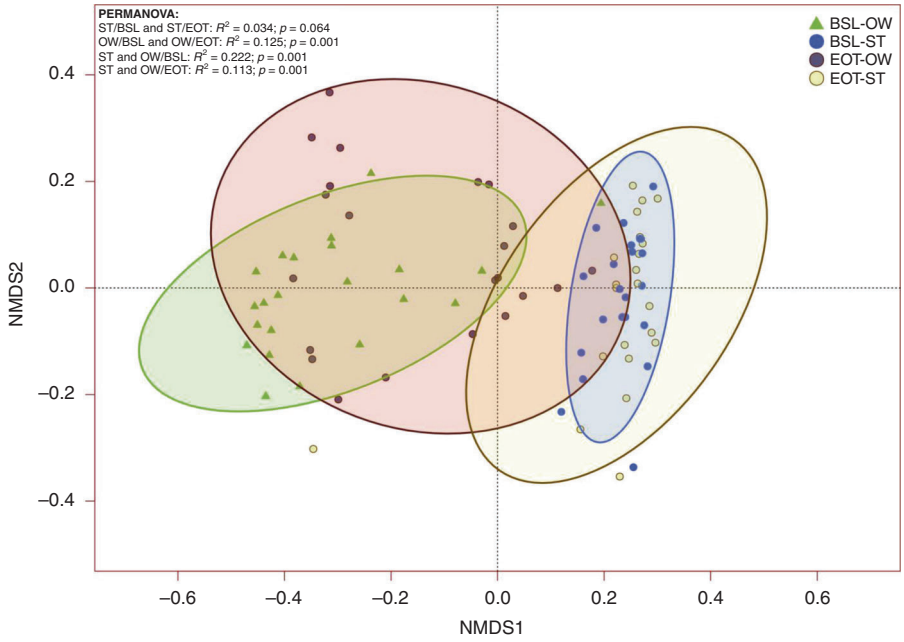

Fig. 4 Impact of chemoradiation therapy on oral and intestinal microbial communities. Non-metric multidimensional scaling ordination plot based on Bray–Curtis dissimilarity with group-specific standard deviational ellipse (90%). CRT chemoradiation, BSL baseline, EOT end of treatment, OW oropharyngeal swabs over the tumour site.

with 100% compliance in sample acquisition and analysis. The stage-specific microbial features in the oral and gut communities from this cohort are hypothesis-generating and should be further investigated to evaluate their use as a biomarker for risk stratification in patients with HPV+ OPSCC. Additional correlation with HPV-related factors such as serotype or viral load in saliva and comparison with a matched-HPV-negative cohort are to be explored. These findings might serve as a 'control' for the microbiome landscape as therapeutic interventions such as immunotherapy are being incorporated into the treatment of these patient populations. Indeed, prospective evaluation of oral and intestinal microbiome is currently ongoing in the setting of an international prospective chemo-sparing approach evaluating definitive chemoradiation vs immunoradiotherapy in HPV+ intermediate-risk OPSCC (NCT03410615). The transitions observed in the composition of the oral but not gut microbiome following treatment might not only have prognostic value but also therapeutic implications to explore gut microbiome modulation strategies in this setting. In this regard, we are currently evaluating the feasibility of gut microbiome intervention in the context of CRT in patients with LA-OPSCC using an oral consortium of taxa associated with immune checkpoint inhibitor-responsiveness (NCT03838601).

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AUTHOR CONTRIBUTIONS

M.O., L.L.S. and A.S. developed the concept and design of the ROMA LA-OPSCC study. Patient recruitment and sample collection was performed by M.O., A.S., L.L.S., R.T. and K.T. Clinical data collection, analysis and curation was performed by M.O. P.H.H.S. and M.C. performed the experimental work. P.H.H.S. and M.C. performed the following experiments: 16S qPCR quantification (M.C.), sequencing library preparation, taxonomic profiling (with CAGEF), statistical analyses (P.H.H.S.) and figure generation (P.H.H.S.). M.O., P.H.H.S., L.L.S., B.C. and A.S. wrote the initial manuscript and all authors were involved in the review and finalisation.

ADDITIONAL INFORMATION

Ethics approval and consent to participate ROMA LA-OPSCC-001 was a prospective observational study that only involved the collection and analysis of saliva, oropharyngeal swabs and stool samples and did not determine the eligibility to receive treatment. The study was approved by the Princess Margaret Cancer Centre Institutional Research Ethics Board (Study ID 17-5693) and was conducted in accordance with the Declaration of Helsinki. All patients provided written, signed, informed consent to participate.

Consent for publication Not applicable.

Data availability The data sets generated and/or analysed during the current study are not yet publicly available but are available from the corresponding author on reasonable request.

Competing interests M.O.: consultant for: Bristol-Myers Squibb Canada (compensated), Mirati Therapeutics. Speaker's Bureau for: None. Employee of: None. Grant/Research support from (Clinical Trials): Mirati Therapeutics, Nubyota. L.L.S.: Consultant for: Merck (compensated), Pfizer (compensated), Celgene (compensated), AstraZeneca/Medimmune (compensated), Morphosys (compensated), Roche (compensated), GeneSeeq (compensated), Loxo (compensated), Oncorus (compensated), Symphogen (compensated), Seattle Genetics (compensated), GSK (compensated), Voronoi (compensated), Treadwell Therapeutics (compensated). Speaker's Bureau for: None. Grant/Research support from (Clinical Trials for institution): Novartis, Bristol-Myers Squibb, Pfizer, Boerhinger-Ingelheim, GlaxoSmithKline, Roche/Genentech, Karyopharm, AstraZeneca/Medimmune, Merck, Celgene, Astellas, Bayer, Abbvie, Amgen, Symphogen, Intensity Therapeutics, Mirati, Shattucks, Avid. Stockholder in: Agios (spouse). Employee of: None. A.S.: consultant for: Merck (compensated), Bristol-Myers Squibb (compensated), Novartis (compensated), Oncorus (compensated). Speaker's Bureau for: None. Grant/Research support from (Clinical Trials): Novartis, Bristol-Myers Squibb, Symphogen AstraZeneca/Medimmune, Merck, Bayer, Surface Oncology, Northern Biologics, Janssen Oncology/Johnson & Johnson, Roche, Array Biopharma. Stockholder in: None. Employee of: None. All of the other authors declare that they have no competing interests.

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Supplemental information belonging to:

Transitions in oral and gut microbiome of HPV+ oropharyngeal squamous cell carcinoma following definitive chemoradiotherapy (ROMA LA-OPSCC study)

Article type**:** Original Article/Research.

Authors: Marc Oliva[1*] and Pierre H.H. Schneeberger[2*], Victor Rey[2], Rachel Taylor[1], Aaron R. Hansen[1], Kirsty Taylor[1], Andrew Bayley[3], Andrew J Hope[3], Ali Hosni Abdalaty[3], Scott V. Bratman[3], Jolie Ringash[3], Ilan Weinreb[4], Bayardo Perez-Ordonez[4], Ralph Gilbert[5], John Waldron[3], Wei Xu[6], David Guttman[7], Lillian L. Siu[1], Bryan Coburn[2**] and Anna Spreafico[1**].

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Supplementary Figure Captions

Supplementary Figure 1. ROMA LA-OPSCC study design.

Supplementary Figure 2. Flow diagram of patients enrolled and included in the analysis.

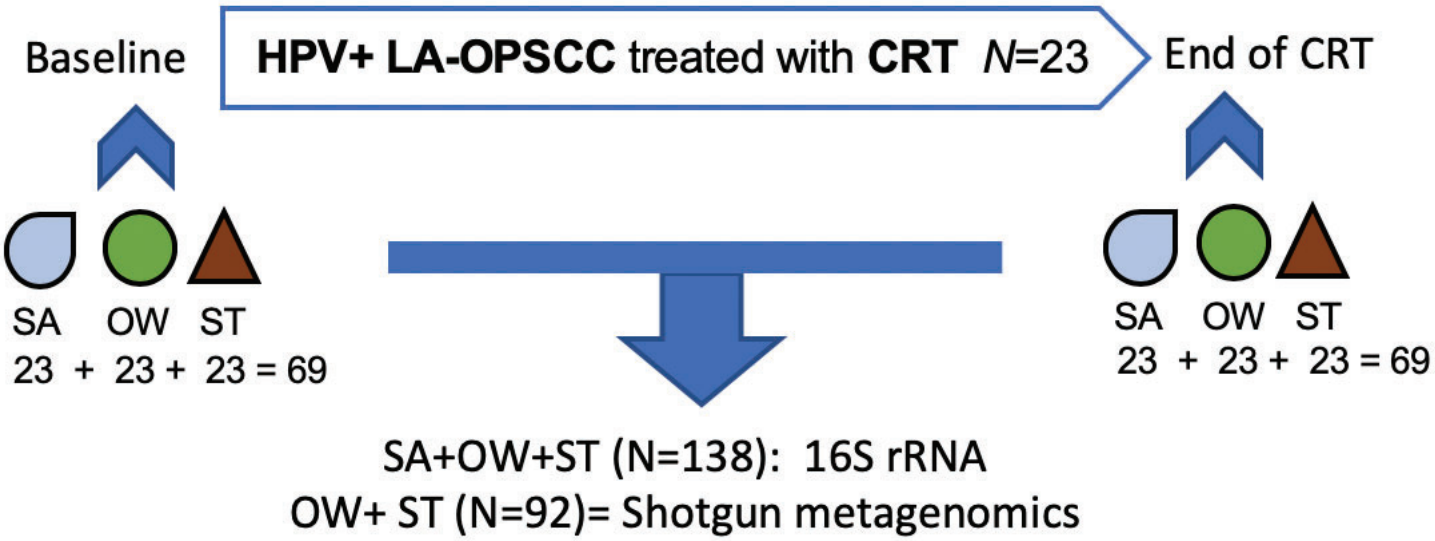
Supplementary Figure 3. **Oral microbiome composition using saliva vs Oropharyngeal swabs. A**. Compositional dissimilarity between saliva and oropharyngeal samples based on 16S rRNA gene sequencing. **B**. Intra-individual diversity compared to inter-patient diversity using Bray-Curtis dissimilarity as a distance measure.

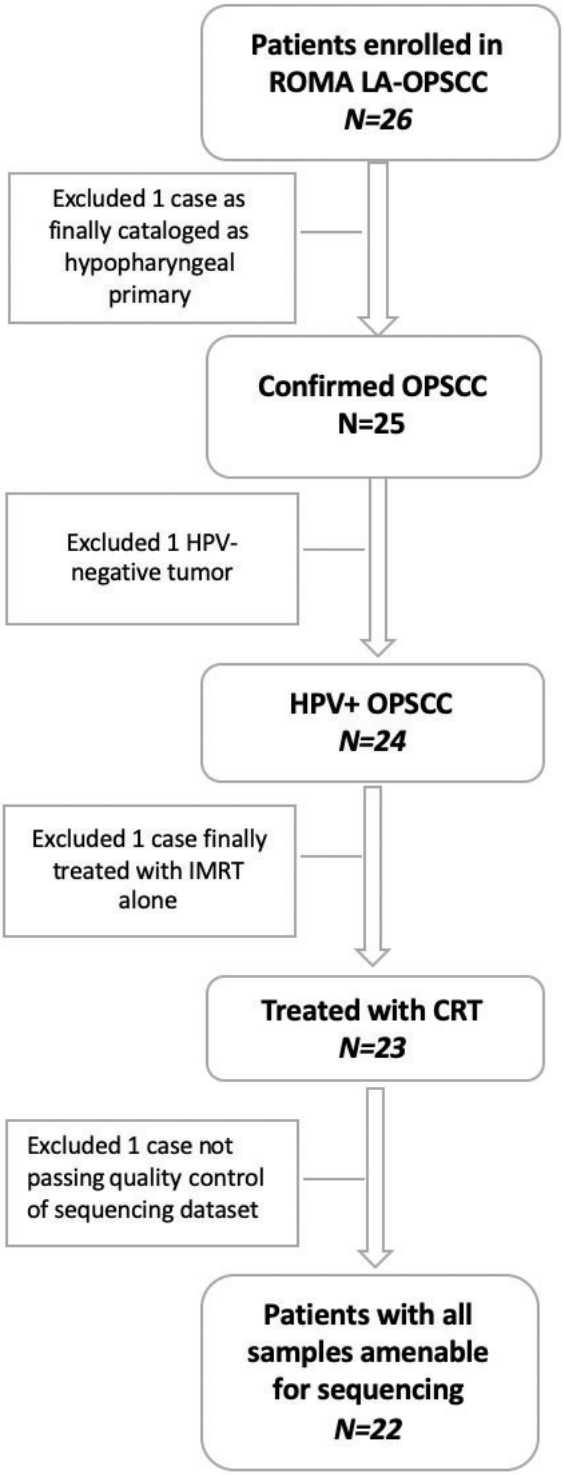
Supplementary Figure 4. Impact of chemoradiation therapy on oral and intestinal microbial diversity. Alpha diversity indices (upper left, lower left and right panels) and bacterial density (upper right panel) comparison in oral communities at baseline and post-CRT.

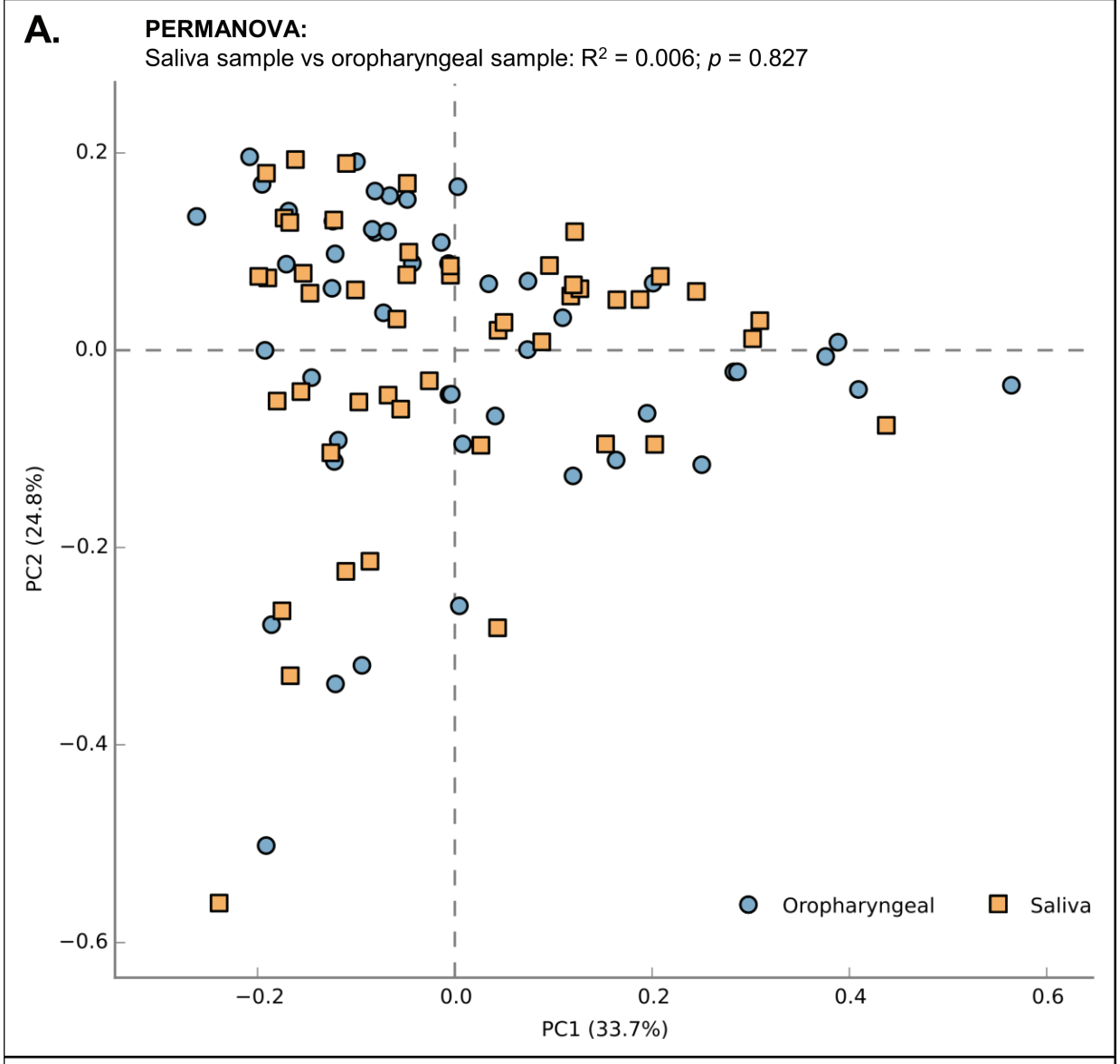
Supplementary Figure 5. Effect of sequencing depth on various indices used for taxonomic as well as functional profiling of microbial communities. A-E. Scatter plot showing the associations between several metrics used for taxonomic and functional analyses and sequencing depth. **F.** Spearman correlation coefficient and statistical significance between sequencing depth and taxonomic and functional metrics. A lack of correlation indicates that there is no bias introduced by sequencing depth whereas a correlation indicates that results are confounded by sequencing depth.

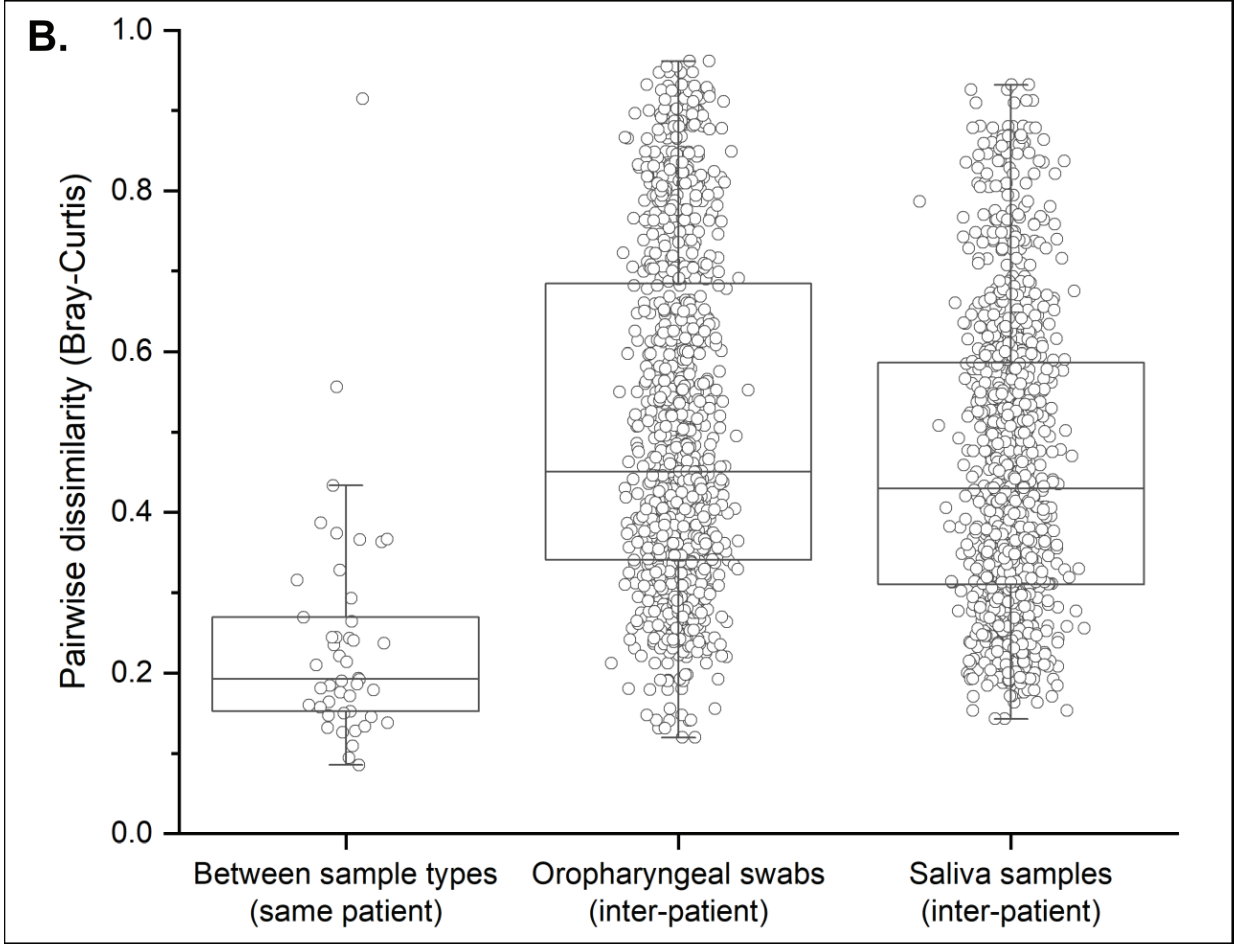
Supplementary Figure 6. Effect of chemoradiation therapy (CRT) on the intestinal microbiome. A. Heatmap showing sample taxonomic composition and clustering based on Bray-Curtis dissimilarity. **B.** Alpha diversity indices stratified by collection time, including species number (left panel), Shannon diversity (middle panel), and Berger-Parker dominance (right panel). The *P*-value is calculated using a non-parametric Wilcoxon test.

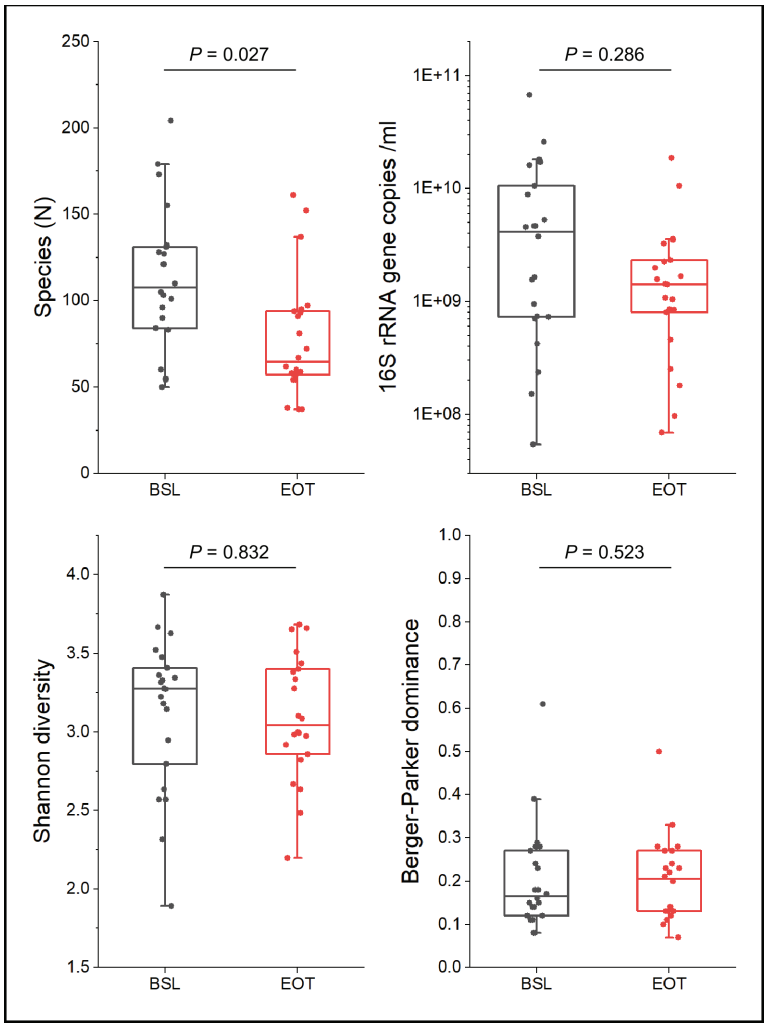
Supplementary Figure 7. Gut communities in post-treatment stool samples based on the use of antibiotics and the route of administration.

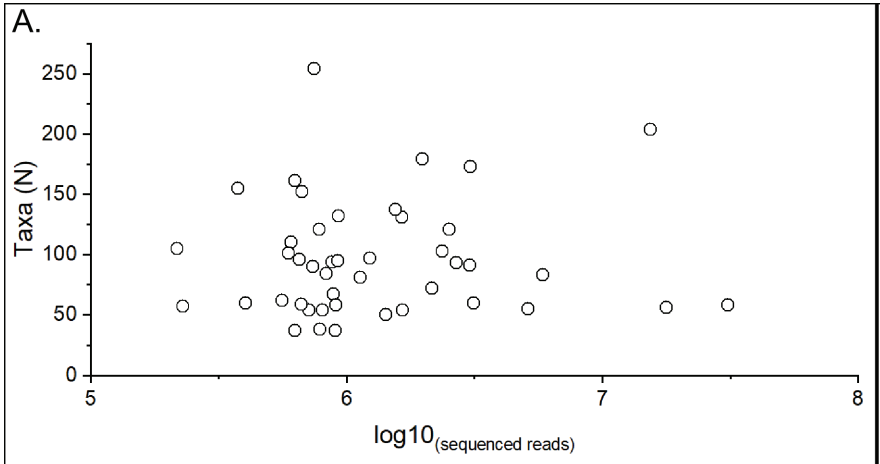

Baseline
HPV+ LA-OPSCC treated with CRT N=23
End of CRT
SA OW ST
23 + 23 + 23 = 69
SA OW ST
23 + 23 + 23 = 69
SA+OW+ST (N=138): 16S rRNA
OW+ ST (N=92)= Shotgun metagenomics

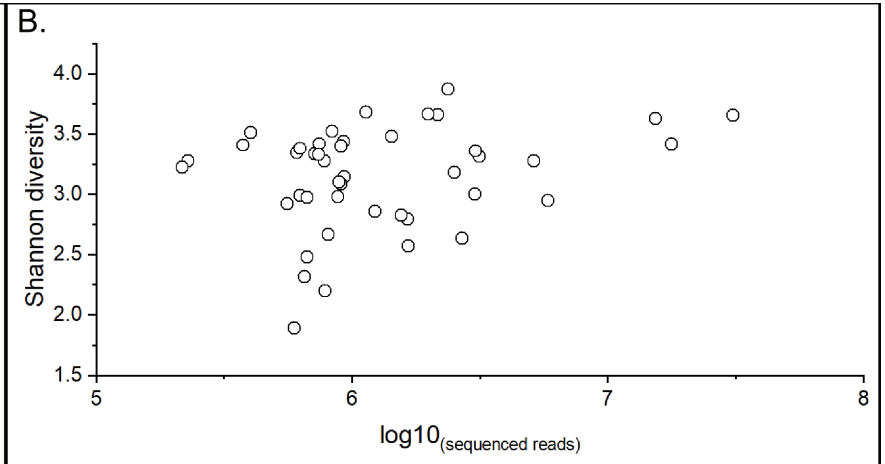

Patients enrolled in ROMA LA-OPSCC N=26
Excluded 1 case as finally cataloged as hypopharyngeal primary
Confirmed OPSCC N=25
Excluded 1 HPV-negative tumor
HPV+ OPSCC N=24
Excluded 1 case finally treated with IMRT alone
Treated with CRT N=23
Excluded 1 case not passing quality control of sequencing dataset
Patients with all samples amenable for sequencing N=22

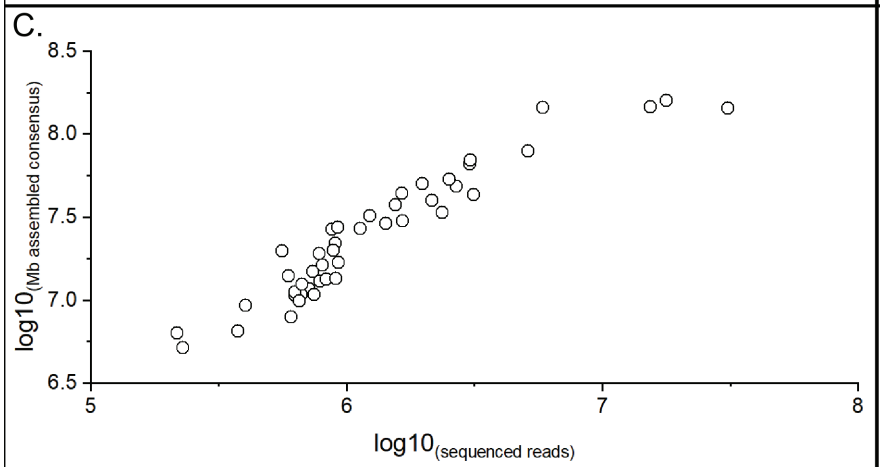

A.
PERMANOVA:
Saliva sample vs oropharyngeal sample: R^2 = 0.006; p = 0.827
PC2 (24.8%)
PC1 (33.7%)
Oropharyngeal
Saliva

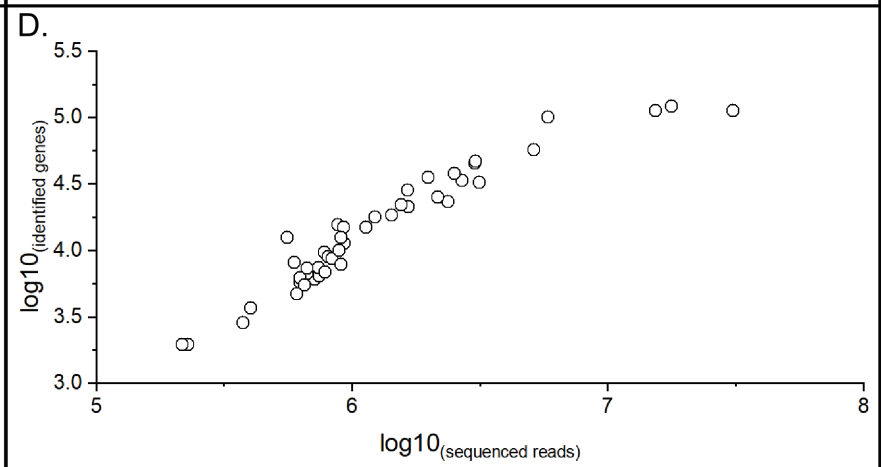

B.
Pairwise dissimilarity (Bray-Curtis)
Between sample types (same patient)
Oropharyngeal swabs (inter-patient)
Saliva samples (inter-patient)

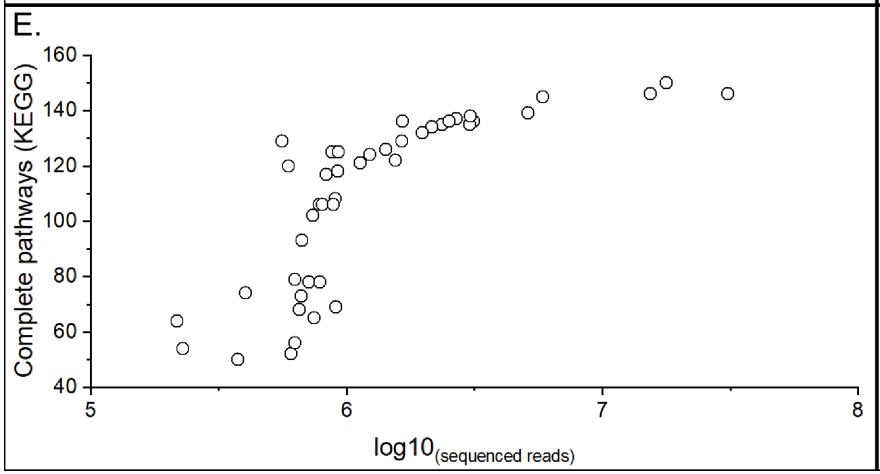

P = 0.027
Species (N)
250
200
150
100
50
0
BSL
EOT
P = 0.286
16S rRNA gene copies /ml
1E+11
1E+10
1E+09
1E+08
BSL
EOT
P = 0.832
Shannon diversity
4.0
3.5
3.0
2.5
2.0
1.5
BSL
EOT
P = 0.523
Berger-Parker dominance
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
BSL
EOT





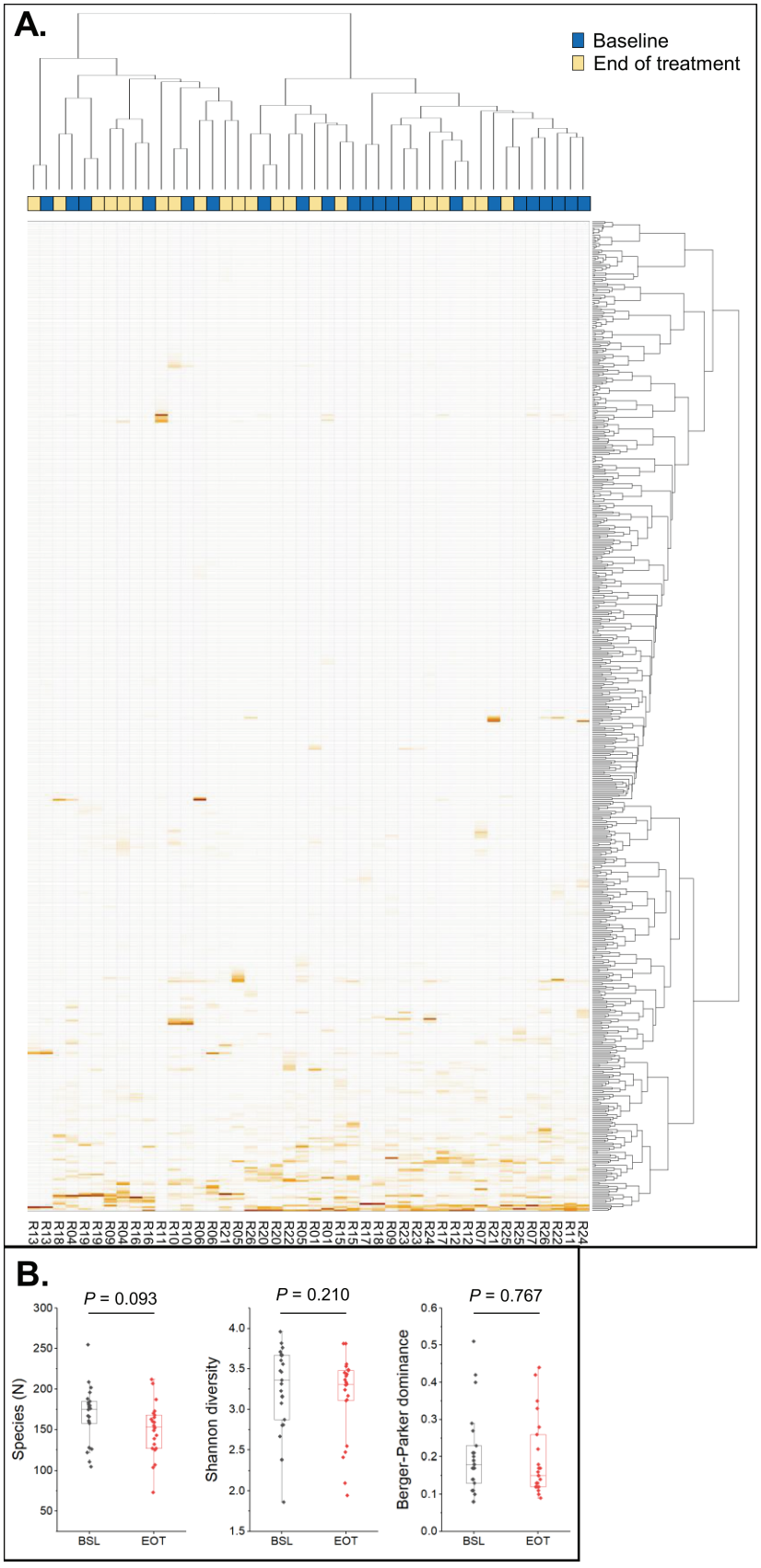


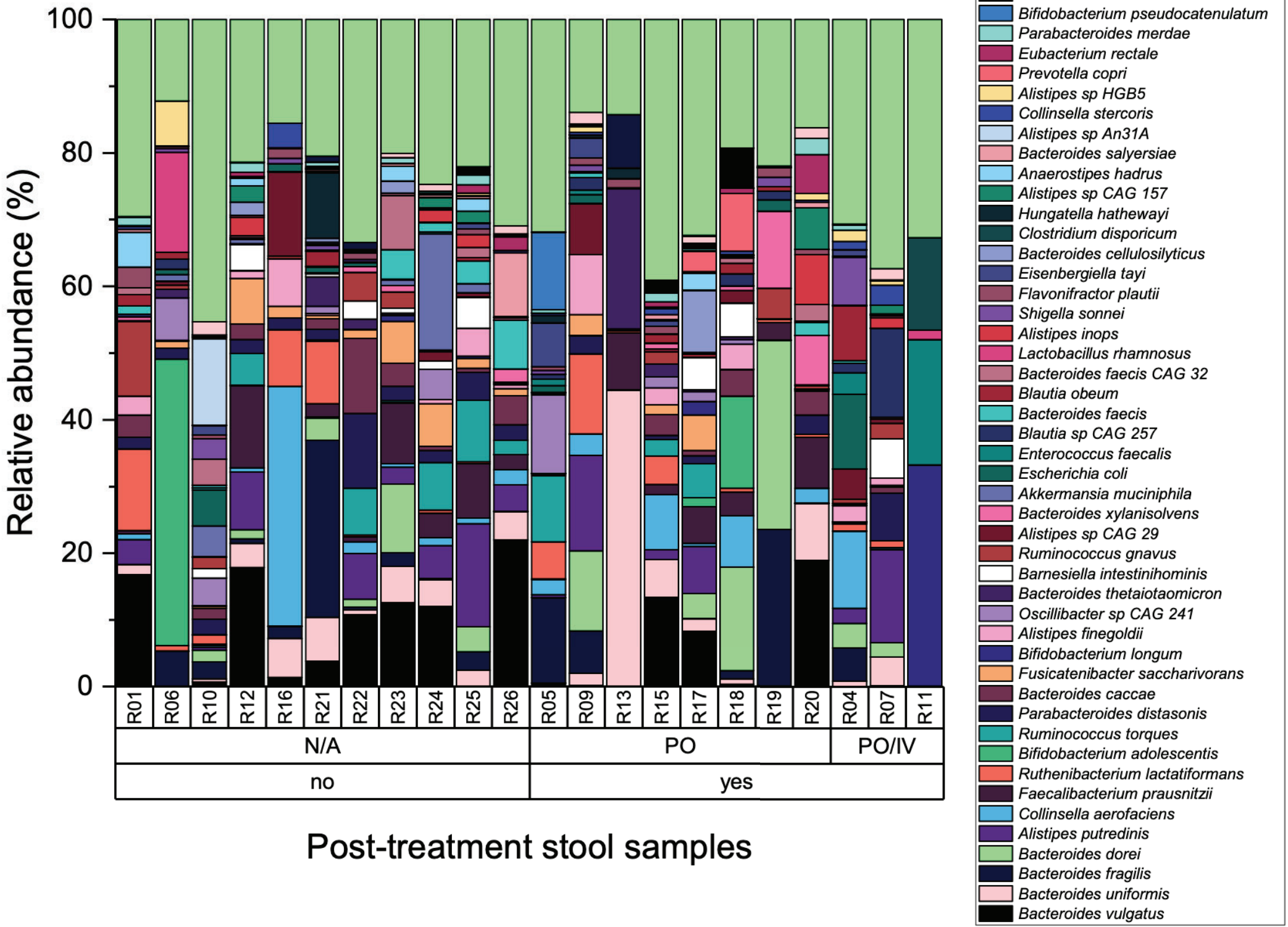




F.

	Metrics	Sequenced reads (Spearman correlation)	P-value
Taxonomic analysis	Taxa (N)	-0.005	0.977
	Shannon diversity	0.208	0.175
Functional analysis	Mb assembled consensus	0.893	<0.0001
	Identified genes	0.947	<0.0001
	Complete pathways (KEGG)	0.951	<0.0001


A.
Baseline
End of treatment
R13 R13 R18 R04 R19 R19 R09 R04 R16 R16 R11 R10 R10 R06 R06 R21 R05 R26 R20 R20 R22 R05 R01 R01 R15 R15 R17 R18 R09 R23 R23 R24 R17 R12 R12 R07 R21 R25 R25 R07 R26 R22 R11 R24
B.
P = 0.093
P = 0.210
P = 0.767
Species (N)
Shannon diversity
Berger-Parker dominance
BSL
EOT
BSL
EOT
BSL
EOT


Relative abundance (%)
100
80
60
40
20
0
R01
R06
R10
R12
R16
R21
R22
R23
R24
R25
R26
R05
R09
R13
R15
R17
R18
R19
R20
R04
R07
R11
N/A
PO
PO/IV
no
yes
Post-treatment stool samples
Others
Bacteroides thetaiotaomicron CAG 40
Desulfovibrio piger
Eubacterium hallii
Bifidobacterium pseudocatenulatum
Parabacteroides merdae
Eubacterium rectale
Prevotella copri
Alistipes sp HGB5
Collinsella stercoris
Alistipes sp An31A
Bacteroides salyersiae
Anaerostipes hadrus
Alistipes sp CAG 157
Hungatella hathewayi
Clostridium disporicum
Bacteroides cellulosilyticus
Eisenbergiella tayi
Flavonifractor plautii
Shigella sonnei
Alistipes inops
Lactobacillus rhamnosus
Bacteroides faecis CAG 32
Blautia obeum
Bacteroides faecis
Blautia sp CAG 257
Enterococcus faecalis
Escherichia coli
Akkermansia muciniphila
Bacteroides xylanisolvens
Alistipes sp CAG 29
Ruminococcus gnavus
Barnesiella intestinihominis
Bacteroides thetaiotaomicron
Oscillibacter sp CAG 241
Alistipes finegoldii
Bifidobacterium longum
Fusicatenibacter saccharivorans
Bacteroides caccae
Parabacteroides distasonis
Ruminococcus torques
Bifidobacterium adolescentis
Ruthenibacterium lactatiformans
Faecalibacterium prausnitzii
Collinsella aerofaciens
Alistipes putredinis
Bacteroides dorei
Bacteroides fragilis
Bacteroides uniformis
Bacteroides vulgatus

Supplementary Table 1. Cohort characteristics.

Variable	N = 22
Median age (range)	61 (range 50-71)
Sex (%)	
Male	19 (86)
Female	3 (14)
Smoking status (%)	
Current	4 (17)
Former	10 (48)
Never-smoker	8 (35)
Smoking pack-years (%)	
<10	10 (45)
≥10	12 (55)
Primary (%)	
Base of tongue	7 (32)
Tonsil	13 (59)
Soft palate	2 (9)
T 8th Ed. (%)	
T0-2	10 (46)
T3	6 (27)
T4	6 (27)
N 8th Ed. (%)	
N0	4 (18)
N1-2	15 (68)
N3	3 (14)
Stage AJCC 8th ed. (%)	
I	6 (28)
II	8 (36)
III	8 (36)
Tooth extraction prior CRT	
Yes	5 (23)
No	9 (41)
Unknown	8 (36)
Radiation completion (%)	
Yes	22 (100%)
No	0
Cisplatin dose (%)	
<200 mg/m^2	6 (28)
=200 mg/m^2	8 (36)
>200 mg/m^2	8 (36)
Use of antibiotics 1-month prior or during CRT	
Yes	11 (50%)
No	11 (50%)
Median duration (range)	8 days (4-15)
Type of antibiotics	
Penicillins	4 (36)
Cephalosporins	5 (45)
Macrolides	2 (19)
Fluorquinolones	1 (1)
Route of antibiotic administration	
Oral	11 (100)
Intravenous	3 (27)

Grade 3/4 Neutropenia **Grade 3/4 Febrile Neutropenia**	4 (17) 1 (4)
Grade 3/4 Mucositis	7 (32)

Supplementary Table 2. Effect of potential confounders on the oral and stool microbiota at baseline measured using a PERMANOVA analysis.

Oropharyngeal samples	R-squared	*P*-value
Smoking status (Current/Former/Never)	0.084	0.498
Tumour location (Base of tongue/Soft palate/Tonsil)	0.096	0.315
Stage (I/II/III)	0.110	0.198
T-staging (T1-4)	0.180	0.062

Stool samples	R-squared	*P*-value
Smoking status (Current/Former/Never)	0.107	0.204
Tumour location (Base of tongue/Soft palate/Tonsil)	0.069	0.903
Stage (I/II/III)	0.089	0.519
T-staging (T1-4)	0.125	0.701

Supplementary Table 3: Effect of antibiotics on the composition in post-treatment stool samples

Variable	R-squared	*P*-value
Antibiotics	0.05	0.33
Route of administration	0.063	0.113

Supplementary Table 4. Interaction between potential confounders and CRT

Oropharyngeal samples	R-squared	*P*-value
Collection time	**0.118**	**0.001**
Collection time vs Smoking status (Current/Former/Never)	0.069	0.894
Collection time vs Tumour location (Base of tongue/Soft palate/Tonsil)	0.074	0.816
Collection time vs Stage (I/II/III)	0.076	0.755
Collection time vs T-staging (T1-4)	0.147	0.353
Collection time vs Mucositis (G1-3)	0.111	0.156
Collection time vs Antibiotics (Yes/No)	0.084	0.631
Collection time vs G-tube dependency at FU	0.047	0.407

Stool samples	R-squared	*P*-value
Collection time	0.034	0.146
Collection time vs Smoking status (Current/Former/Never)	0.110	0.363
Collection time vs Tumour location	0.078	0.964
Collection time vs Stage (I/II/III)	0.093	0.775
Collection time vs T-staging (T1-4)	0.121	0.956
Collection time vs Mucositis (G1-3)	0.119	0.200
Collection time vs Antibiotics (Yes/No)	0.089	0.821
Collection time vs G-tube dependency at FU	0.043	0.791

Article 2. Prospective manipulation of the gut microbiome with Microbial Ecosystem Therapeutic 4 (MET-4) in HPV-related locoregionally-advanced oropharyngeal cancer squamous cell carcinoma (LA-OPSCC) undergoing primary chemoradiation: ROMA2 study

Authors: Marc Oliva, Alya Heirali, Geoffrey Watson, Ashley M. Rooney, Kyla Cochrane, Sarah Jennings, Rachel Taylor, Minge Xu, Ali Hosni, Andrew Hope, Scott V. Bratman, Douglas Chepeha, Ilan Weinreb, Bayardo Perez-Ordonez, Ricard Mesia Nin, John Waldron, Wei Xu, Aaron Hansen, Lillian L Siu, Bryan Coburn and Anna Spreafico.

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