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Review

Potential role of microbiome in oncogenesis, outcome prediction and therapeutic targeting for head and neck cancer

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ABSTRACT

In the last decade, human microbiome research is rapidly growing involving several fields of clinical medicine and population health. Although the microbiome seems to be linked to all sorts of diseases, cancer has the biggest potential to be investigated.

Following the publication of the National Institute of Health - Human Microbiome Project (NIH-HMP), the link between Head and Neck Cancer (HNC) and microbiome seems to be a fast-moving field in research area. However, robust evidence-based literature is still quite scarce. Nevertheless the relationship between oral microbiome and HNC could have important consequences for prevention and early detection of this type of tumors.

The aims of the present review are: (i) to discuss current pre-clinical evidence of a role of oral microbiome in HNC; (ii) to report recent developments in understanding the human microbiome's relationship with HNC oncogenesis; (iii) to explore the issue of treatment response and treatment toxicity; (iv) to describe the role of microbiota as potentially modifiable factor suitable for targeting by therapeutics.

Further studies are needed to better establish the causal relationship between oral microbiome and HNC oncogenesis. Future trials should continue to explore oral microbiome in order to build the scientific and clinical rationale of HNC preventative and ameliorate treatment outcome.

Introduction

Head and neck cancer (HNC) is a rare and heterogeneous disease with disparities across Europe and the United States in term of incidence and survival rates [1,2]. According to the EUROCARE-5 population-based study, the incidence rate of larynx (LC) (4.6/100,000), oral cavity (OCC) (3.5/100,000) and oropharynx (OPC) (3.3/100,000) sub-sites is higher than that of nasopharyngeal carcinoma (NPC) (0.47/100,000). Despite combined treatment, including surgery, radiotherapy and chemotherapy or combinations of these therapeutic modalities, 5-year overall survival rates range from 25% to 60% and mainly depend on primary site at diagnosis [2].

In addition to tobacco smoking and alcohol use, human papillomavirus (HPV) type 16 and Epstein-Barr virus (EBV) infections have been correlated with the onset of OPC and NPC, respectively [3,4]. In most cases HPV- related OPCs represent a distinct clinical and biological entity and have an improved prognosis compared to HPV-negative disease [3]. Whereas high EBV deoxyribo-nucleic acid (DNA) levels are strictly correlated to worse prognosis than NPC patients with low levels of EBV DNA [5,6].

Nowadays, other potential biomarkers are under investigation to propose a patient-centered approach in order to predict clinical outcome and develop a biomarker-guided treatment [7]. Over the years, several factors, such as neutrophil to lymphocyte ratio, circulating tumor cells (CTCs) and programmed death-ligand 1 (PD-L1) expression in human tissue, have been identified but their definitive implementation in daily clinical practice is still pending [8–12].

An effective definition of biomarkers able to predict both survival outcomes and RT-related side effects represents an attractive priority in HNC field. In addition to the well-known clinical and dosimetric factors,

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also the individual radio-sensitivity – based on human genetic profile – appear related to side-effects risk [13,14]. But definitive data are still lacking [15].

Following the publication of the National Institute of Health - Human Microbiome Project (NIH-HMP), the link between HNC and microbiome seems to be a fast-moving field in research area [16]. This interplay can be promoted by intrinsic oncogenic properties, mucosal inflammatory conditions, metabolic or immune deregulations, anti-cancer immunity modulation or the effectiveness of anticancer therapy [17–21].

Here, we presented a review to discuss current pre-clinical and clinical evidence of microbiome in HNC. Firstly, the role of human microbiota in the oncogenesis is examined and a focus on HNC is presented. Secondly, we looked at the issue of treatment response and treatment toxicity, mainly from radiotherapy perspectives. Finally, evidence from clinical studies is reported and the potential for future therapeutic strategies is considered.

Literature search strategy was conducted using PubMed and Scopus databases, based on the following combinations of key words: “microbiota”, “microbiome”, “bacteria”, “composition”, “healthy ecosystem”, “probiotics”, “carcinogenesis”, “mucosa”, “mucositis”, “nasopharyngeal”, “oropharyngeal”, “laryngeal”, “oral cavity”, “pharyngeal” “head and neck”, “cancer”. Search was limited to papers published in English. References of the included studies were carefully reviewed for relevant papers that would have been missed by electronic search. Hand searching (meeting proceedings of European Society of Medical Oncology and European SocieTy for Radiotherapy & Oncology) and clinicaltrials.gov were also used. Search strategy was performed up to June 2019.

Human microbiome and oncogenesis

Firstly coined in 2001, the term *microbiota* describes the entire ecological community, including commensal, symbiotic and pathogenic microorganisms that inhabit human body in health and disease [22].

Although the terms *microbiota* and *microbiome* are frequently used interchangeably, it is useful to specify that the term *microbiome* has a wider meaning, representing ≪ the genes and genomes of the microbiota, as well as the products of the microbiota and the host environment ≫, and, therefore, it includes plasmid DNA, viruses, archaea and fungi – even though these are not commonly detected – [23]. To note archaea refer to single-celled microorganisms able to live in the extreme environments (very high (over 80 °C) or very low temperatures, or highy salty, acidic or alkaline water). They are obligate anaerobes and have distinct structure and metabolism from bacteria and eukaryotes. In particular, unlike bacteria, no archaea produce spores and can do photosynthesis [23,24].

Microorganisms identification derived from culture-based approaches, using the 16 Svedberg unit (S) rRNA gene technology for bacteria, the 18S rRNA gene sequencing technique for eukaryotic microbes and genomic regions for viruses [24]. Technical background and detailed information of sequencing protocols are adequately covered in specific literature and we refer interested readers to those papers because these topics are beyond the scope of this review [25,26].

Over the years, a vast community of bacteria, archaea, eurkaryotes and viruses has been detected in human body [27]. All these microorganisms constantly maintain the homeostasis between local environment and immune host, primarily in oral cavity, gastrointestinal tract, vagina and skin [28,29]. Highly assorted microbial communities are usually related to a health status. Whereas, shifts in the commensal equilibrium generally define a pathological inflammation that can lastly promote tumor growth [29]. Surely cancer onset is a complex and multi-factorial entity. Changes in human microbiome could determine an oncogenic effect by (i) induce chronic inflammatory response, (ii) instigate cellular anti-apoptotic signals, (iii) release carcinogenic factors and (iv) modulation of anti-cancer immunity [30]. The right mixture of these direct – manipulation of host cell biology – and indirect – chronic inflammation – effects might contribute to carcinogenesis [31]. For instance, Helicobacter pylori, a gram-negative bacterium, is a well-definite cause of gastric cancer, both adenocarcinoma and mucosa associated lymphoid tissue lymphoma. Secondary to chronic inflammation, due to up-regulation of cyclooxygenase-2 (COX2) expression, cytokines and reactive oxygen species and nitric oxide intermediates, Helicobacter pylori induces an oxidative DNA damage to the gastric mucosa, as well as a progressive structural change, including mucous barrier degradation and increased cell turnover [32]. This direct and indirect perturbation of microenvironment is crucial to induce carcinogenesis [32]. Other strong association between specific bacterial infection and cancer promotion has been demonstrated between Chlamydia pneumoniae and lung cancer, Salmonella typhi and gallbladder cancer, Streptococcus bovis and colon cancer, Propionibacterium acnes and prostate cancer [31]. Similarly, viral infections, such as hepatitis B virus (HBV), EBV and HPV have been shown to cause cancer, due to initiating (through DNA integration into the human genome) and promoting capabilities [33]. The most convincingly associations are HBV and hepatocellular carcinoma, EBV and non-Hodgkin's lymphoma, Hodgkin's lymphoma and NPC, and HPV and cervical cancer and OPC [33].

Focus on oral/laryngeal/pharyngeal microbiome

A comprehensive description of oral microbiome represents a logical step to value healthy state or pathological state of oral mucosal cavity. The oral cavity is an ideal site of microorganisms growth because of its temperature (37 °C), its pH (6.5–7.5) and its soft (tongue, palate, buccal mucosa) and hard surfaces (teeth). In fact, accounting more than 700 species, oral cavity is one of the most densely populated human microbiome habitats [34]. Oral microbial community colonization begins at birth, evolves during childhood and its definitive structure is established during youth/adulthood [35]. Individual variations are mostly related to subjective immune, environmental and dietary exposures [36]. Table 1 listed the predominant microorganisms in the healthy human oral cavity [34,37,38]. Bacterial flora, including species of Gemella, Granulicatella, Streptococcus and Veillonella, is the most represented [37]. The vast majority of oral habitats are dominated by Streptococcaceae species [37]. Oral archaea – Methanobrevibacter smithii, Methanobrevibacter oralis and Methanosphaera stadtmanae – are often detected in periodontal pockets and dental calculus and favor the development of fermenting bacteria phyla [39]. Whereas Candida

Table 1
Main species in healthy oral microbiome.

Species	Location					
	Gengival cervice	Tooth surface	Tongue	Palate	Buccal mucosa	Saliva
Bacteria						
Streptococcus	x	x	x	x	x	x
Gemella	x	x	x	x	x	
Lactobacillus	x	x	x			x
Moraxella			x			x
Actinomyces	x	x	x			
Veillonella	x		x			x
Campylobacter	x					
Granulicatella		x	x	x	x	
Neisseria		x		x		
Fusobacterium	x					
Prevotella	x		x			
Fungi						
Candida		x			x	
Cladosporium					x	
Aureobasidium					x	
Saccharomycetales					x	
Archaea		x				

Table 2
Oral micobiome: main findings in oral/oropharyngeal squamous cell carcinoma.

Author	Study			Predominant species in cancer
	Type	Population	#patients	
Sasaki et al. [53]	Retrospective	OC	49	Streptococcus anginosus
Mager et al. [54]	Retrospective	OC	344 (45 OC vs 299 control)	Capnocytophaga gingivalis, Prevotella melaninogenica, Streptococcus mitis
Katz et al. [55]	Retrospective	OC	15 (10 OC vs5 control)	Porphyromonas gingivalis
Pushalkar et al. [52]	Retrospective	OC	20 (10 OC vs 10 control)	Streptococcus (S. oral, S. salivarius, S. gordonii, S. parasanguinis), Peptosteptococcus stomatis, Gemella (G. haemolysans, G. morbillorum), Johnsonella ignava
Guerrero-Preston et al. [56]	Retrospective	HNSCC	44 (19 HNSCC vs 25 control)	Lactobacillus, Streptococcus
Schmidt et al. [57]	Retrospective	OC	14 (9 OC vs5 control)	Prevotella (P. intermedia, P. melaninogenica, P. nanceiensis, P. oris, P. tannerae, P. melaninogenica)

: number; OC: oral cavity cancer; HNSCC: head and neck squamous cell carcinoma.

and Cladosporium are the most abundant species that characterizes the fungi component in oral cavity [38]. Both indirect and direct metabolic interactions between these micro-organisms are paramount to establish and maintain a healthy oral microbiome while preventing a shift toward a disease (dysbiosis) [40].

To date the nature of microbial synergy in the human larynx region is not completely understood. In Gong H et al. study [41], a broad range of taxa with different abundances was detected in the larynx of 32 healthy subjects. It included the phyla *Firmicutes* (46.4%), *Bacteroidetes* (18.7%), *Fusobacteria* (16.9%), *Proteobacteria* (13.0%) and *Actinobacteria* (2.4%). Whereas at the genus level, *Streptococcus* (41.7%), *Fusobacterium* (17.0%), *Prevotella* (13.2%), *Gemella* (4.1%), *Helicobacter* (2.6%), and *Haemophilus* (2.3%) were the predominant populations [41].

The influence of microbiome on HNC carcinogenesis

Examine the association between oral microbiome and HNC gives support to the idea that at least some microorganisms can contribute to carcinogenesis process.

As for periodontal disease – Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola are now recognized as frequent cause in periodontal disease [37] –, it could be useful to explore a distinctive bacterial flora specifically associated with OCC and OPC in order to effectively define a prevention program. It has been demonstrated that periodontitis associated to oral HPV co-infection may increase OPC risk, primarily due to epithelial tissue loss and subsequent basal cells infection [42]. Larger epidemiologic studies are needed to confirm this association in the natural OCC and OPC carcinogenesis. For sure, to fully elucidate the influence of oral microbiota in OCC and OPC pathogenesis, other risk factors, including tobacco smoking, alcohol consumption and poor oral hygiene should be investigated concurrently. Epidemiological studies supported the evidence that poor dental status, especially in heavy alcohol consumers and/or smokers, increased OCC risk mainly due to acetaldehyde accumulation [43]. This toxic compound is the first ethanol metabolite and can be produced via bacterial oxidation in high concentrations in saliva by Streptococcus salivarius, alpha-hemolysing Streptococci, Corynebacterium and Stomatococcus [44]. Increased salivary acetaldehyde concentrations have been convincingly shown in alcoholic subjects, following ethanol ingestion, and in smokers, due to the intrinsic tobacco high levels of acetaldehyde [45]. Acetaldehyde may act as a carcinogen because it interferes with DNA synthesis and repair, causing cellular hyper-regeneration, and stimulates oral mucosa inflammation [46]. In this context, an oral microbiota switch to a significant increase in the proportion of these high acetaldehyde producer bacteria could be responsible for OCC cancer. The crosstalk between microbiota and immune system is object of several investigations, especially concerning gut microbiome and its relation to colon-rectal cancer, lung tumors and melanoma. Very few data are available on HNC. Recently, it has been reported a potential role of *Porphyromonas gingivalis* in OCC onset [47]. *Porphyromonas gingivalis* can (i) induce the expression of B7-H1 and B7-DC receptors, (ii) reduce T cell proliferation, (iii) facilitate immune evasion and (iv) promote proenzyme matrix metalloproteinase 9 (proMMP9) expression by activating the ERK1/2-Ets1, p38/HSP27 and PAR2/NF-kB pathways. [47–48].

Over the past 15 years, HPV, especially genotype 16, has been frequently detected in OPC tissue specimens. Characteristically, HPV-related OPC arises in younger patients (aged < 60 years), non-smokers and non-alcohol drinkers, with an early tumor stage and an advanced nodal involvement at diagnosis [49]. This issue gives rise to studies focused on the evaluation of immune risk factors. At present, HPV implications in healthy-appearing oral mucosa and its influence on oral microbiome composition are limited, but HPV infection has been associated with microbiota changes in HPV-related cervical intraepithelial neoplasia and cervical cancer [33,50]. Thus, it is also

expected a possible HPV role in modulating oropharynx pre-cancerous and cancer lesions.

Oral microbiome in OCC and OPC

Even though robust evidence-based literature is quite scarce, it can be stated that tumor microbiome is diverse compared to contiguous healthy oral mucosa. In a comparative evaluation, Nagy et al [51] demonstrated that Candida albicans and anaerobic bacteria – Actinomyces, Clostridium, Fusobacterium, Prevotella, Porphyromonas and members of the Bacteroides ureolyticus/gracilis – were prevalent at tumor surface. Concerning aerobic species, both Gram-negative – Serratia liquefaciens, Klebsiella pneumoniae and Citrobacter freundii – and Gram-positive species – Streptococcus β-haemolyticus and Enterococcus faecalis – were more frequently isolated on tumor site than healthy mucosa [51]. More recently, it has been demonstrated that raised concentrations of saccharolytic and aciduric species, such as Streptococcus mitis/oralis, Ralstonia insidiosa, Gemella haemolysans and Sphingomonas, were significantly more prevalent on tumor surface than non-tumor tissue [52–53]. Table 2 depicts oral microbiome composition implicated in OCC and OPC [53–58]. Considering that most studies have implicated Streptococcus and Prevotella species in OCC and OPC, it is reasonable to assume a plausible causal relationship. Whether the abundance of these micro-organisms has any significance on carcinogenic process remains a concept worthy of further investigation.

Laryngeal/pharyngeal microbiome in LC

The association between the microbiota structure and LC has been poorly investigated. Gong et al compared composition of laryngeal microbiome between LC patients and control population [59]. Microbiota profile in LC patients was significantly different from that in control subjects, suggesting a potential tumor-genesis factor [59]. Interestingly, no difference in bacterial communities was recorded in the microbiota structure of the pharynx in patients with LC and vocal cord polyps [41]. Surely further studies are necessary to better define shifts in laryngeal microbiota associated with malignant profile.

Interplay between oral microbiome and response to immunotherapy

Due to its immunostimulatory function, gut microbiome can improve immunotherapy and chemotherapy efficacy [60]. For instance, its influence on anti-PD-1 activity has been proven in preclinical models, as well as clinical series including patients with melanoma, non small cell lung cancer and renal cell cancer [61–63]. A greater bacterial diversity has been associated to a higher response rates to anti-PD-1 therapy [61–62].

Based on Checkmate 141trial and Keynote 055 trial, nowadays anti-PD-1-antibodies are the standard of care in platinum-refractory HNC patients [64]. Whether the microbiota can predict response to immunotherapy in HNC is still not established. In a Checkmate 141trial sub-analysis, the profile of oral microbiome has been evaluated in order to define possible prognostic biomarkers of response to the anti-PD-1 agent. But no significant correlation between bacterial diversity microbioma (at baseline and one week after therapy) and clinical response was found [65].

Researchers from Princess Margaret Cancer Centre have designed a trial –NCT034106615, still ongoing – to prospectively assess both oral and intestinal microbiota in a homogeneous cohort of HNC patients treated with radical chemoradiotherapy. The aim is to achieve a profound knowledge of microbiota relationship with treatment response.

Interplay between oral micorbiome and therapy-related effects

The introduction of oral microbiome issue in the oncologic field has not only the potential to improve disease knowledge in its broadest sense but also to find a relationship between oral microbiota perturbation and RT-related oral complications. RT plays a crucial role in the treatment of HNC both in definitive and adjuvant settings [49], with or without concomitant chemotherapy according to high risk features. Usually, RT total doses range between 60 and 70 Gy delivered in daily single fraction of 1.8–2.25 Gy [49].

Researches indicate that microorganisms living in the host have a role in initially driving or controlling inflammation from external causes. A dysbiotic milieu may change the metabolism of the host and provoke increased inflammation [66]. Dysbiosis affects the metabolism of short-chain fatty acids, which are known to have immunomodulatory properties and play a part in RT-induced toxicity. While, RT-related toxicity have been abundantly considered over the years, especially due to improvement in RT technique and organ-sparing protocols, minor attention has been directed to basic principles of ionizing radiation/oral microbial interaction [35,67]. Severity of oral injury, such as quantity and composition of saliva, alteration of the oral flora and tooth damage, is mainly related to the radiation dose to the oral cavity region. An exposure of only 20 Gy can cause up to 80% of salivary function reductions [68]. Subsequent oral environment acidification (pH < 5) favors the rise in concentration of acidogenic and cariogenic species, such as Streptococcus mutans, Actinomyces and Lactobacillus, on one hand, and reduces Neisseria, Fusobacterium and Streptococcus sanguinis concentrations, on the other hand. If left uncontrolled, all these caries-related bacteria contribute to the pathogenesis of RT-related dental caries [69]. In addition, Candida albicans tends to profit from these oral microbiota changes and super-infection may occur, both during and after therapy [68]. Surely, education and practices of oral health care should be encouraged in these patients. At present, the translation of oral microbiota as a potential biomarker to predict incidence and severity of oral toxicity is hampered by a lack of definitive evidence of microbiota cells sensitivity to treatment and their contribution to treatment outcome. However, a couple of experiences have been published [69–70]. In a paper by Huo et al, the dynamic variation in oral microbiota during RT and its association to oropharyngeal mucositis progression/aggravation was prospectively evaluated in a cohort of NPC patients [69]. Results showed that the mucosal bacterial alpha diversity (richness and evenness) did not vary significantly during treatment overall. However, several bacteria, including *Prevotella, Fusobacterium, Treponema* and *Porphyromonas*, showed obvious dynamic synchronous shifts in their abundances throughout RT. Their peaks often coincided with the onset of severe mucositis. This suggested that dysbiosis of oral mucosal microbiota may have a role in exacerbating the aggravation of mucositis in NPC patients during RT. Similar results were reported by Zhu et al [70]. The MICRO-LEARNER trial is an ongoing Italian trial (NCT03294122) designed to assess microbial populations evolution in HNC and prostate cancer patients during curative RT and define its impact on host and RT-induced toxicity [71]. In addition, the individual response at the tissue microstructure level, through analysis of images with advanced bioengineering techniques, will be determined. Results from this research, besides suggesting new ways to predict patients at risk of relevant side-effects, may also suggest possible treatments to change the baseline microbiota of patients at high risk or to modify it during therapy, in order to mitigate toxicity.

Indeed, combination of probiotics (primary Lactobacillus species and Bifidobacterium species) and prebiotics (poorly digested oligosaccharides such as arginine) have been investigated for oral microbiota modulation. Probiotics compete with oral micro-organisms for nutrients, produce bacteriocins able to inhibit pathogens growth, modulate cell proliferation/apoptosis and stimulate mucosal immune system. Whereas, prebiotics stimulate the growth of beneficial microorganisms and suppress the activity of the deleterious ones [72]. Due to their ability to selectively stimulate probiotics, the combination prebiotics/probiotics can potentially provide a better oral health condition. Actually, discrepant results are published in literature concerning their

Table 3
Clinical trials investigating prebiotics/probiotics/factor with probiotic effect in RT-related oral mucositis.

Author	Study						
	Type	Population	#patients	Investigation	Treatment	Primary end-point	Results
Sharma et al. [73]	monocenter, randomised, phase III	HNSCC stage II-IVa	188	L. brevis CD2 (93) vs placebo (95)	CRT	severe OM incidence	Significant reduction in severe OM onset (52% vs 77%)
De Sanctis et al. [74]	multicenter, randomized, phase III	HNSCC stage II-IVa	68*	L. brevis CD2 (32) vs sodium bicarbonate (36)	CRT	severe OM incidence	No difference in severe OM onset (40.6% vs 41.6%)
Brizel et al. [75]	multicenter, randomized, phase II	HNSCC stage III-IVb	99	palifermin (67) vs placebo (32)	CRT	grade ≥ 2OM duration	Shorter OM duration (6.5 w vs 8.1 w); lower incidence of severe OM (66% vs 81%)
Jiang et al. [76]	monocenter, randomized,	NPC	99	Probiotic (64) vs placebo (35)	CRT	severe OMincidence	Significant reduction in severe OM onset (15.5% vs 45.7%)

* Enrollment prematurely stopped due to technical and administrative problems (planned accrual: 106 patients).
# : number; HNSCC: head and neck squamous cell carcinoma; L. brevis CD2: Lactobacillus brevis; CRT: chemoradiotherapy; OM: oral mucosistis; w: weeks; RhEGF: recombinant human epidermal growth factor; GM-CSF: granulocyte-macrophage colony stimulating factor; NPC: nasopharyngeal carcinoma.

efficacy and safety in oral disease management, both in prevention and treatment phase [73–74]. Table 3 summarizes those trials specifically designed to test the efficacy of prebiotics/probiotics/factor with probiotic effect in RT-related oral mucositis [73–76]. The major bias is studies heterogeneity, mainly related to different probiotic therapy, end-points and measures. Nutritional interventions, such as oral glutamine and honey, were proposed to prevent or treat RT-induced oral mucositis but also their efficacy were doubtful [77]. Surely, prospective clinical trials are needed to define the value of oral microbiota in HNC initiation and treatment response and the role of factor with probiotic effect in RT-related toxicity modulation. At present, the use of prebiotics/probiotics/factor with probiotic effect cannot be considered part of standard practice in HNC management.

Window-of-opportunity studies

Future trials should continue to explore oral microbiome in order to build the scientific and clinical rationale of HNC preventative and ameliorate treatment toxicity. In fact, both early diagnosis and oral mucositis prevention are relevant factors that impinge on patient quality of life. Prospective population trial to observe the relationship between oral microbiome and its function in HNC should be designed. The identification of a specific link could provide a microbiome-based research paradigm, possibly leading to new preventive programs and treatment approaches in HNC. Changes in microbiota composition might have the potential to be used as a biomarker, but translating oral microbiome research into clinical practice should require incorporation of microbiome surveillance into clinical trials. Moreover, identify a microbiome signature to potentially define different classes to predict cancer risk and survival should be a common purpose. Attempts to develop a model to predict RT-related oral mucositis based on individual radiosensitivity could be appropriate to lastly personalize treatment. In order to ensure appropriate comparisons of trial results, at least the standardization of oral microbiome sampling and the quality of time-to-event measures should be assessed.

Conclusions

We summarized the current evidence concerning the possible link between oral microbiome and HNC and the mechanisms involved. This relationship could have important consequences for HNC prevention and early detection. But, at present conclusions are far from definitive. Further studies are needed to better establish the causal relationship between oral microbiome and HNC management.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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