Oral microbiota and vitamin D impact on oropharyngeal squamous cell carcinogenesis: a narrative literature review

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Review

Title

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Abstract

An emerging body of research is revealing the microbiota pivotal involvement in determining the health or disease state of several human niches, and that of vitamin D also in extra-skeletal regions. Nevertheless, much of the oral microbiota and vitamin D reciprocal impact in oropharyngeal squamous cell carcinogenesis (OPSCC) is still mostly unknown.

On this premise, starting from an *in-depth* scientific bibliographic analysis, this narrative literature review aims to show a detailed view of the state of the art on their contribution in the pathogenesis of this cancer type.

Significant differences in the oral microbiota species quantity and quality have been detected in OPSCC affected patients; in particular, mainly high-risk human papillomaviruses (HR-HPVs), *Fusobacterium nucleatum, Porphyromonas gingivalis*, *Pseudomonas aeruginosa* and Candida spp. seem to be highly represented.

Vitamin D prevents and fights infections promoted by the above identified pathogens, thus confirming its homeostatic function on the microbiota balance. However, its antimicrobial and antitumoral actions, well-described for the gut, have not been fully documented for the oropharynx yet.

Deeper investigations of the mechanisms that link vitamin D levels, oral microbial diversity and inflammatory processes will lead to a better definition of OPSCC risk factors for the optimization of specific prevention and treatment strategies.

Keywords:

Bacterial, fungal and epitheliotropic viral oral pathogens, oral microbiota, oropharyngeal squamous cell carcinoma ~~(OPSCC)~~, vitamin D, health.

INTRODUCTION

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common malignancy in the world with 600,000 new cases/year [1]. Although it mainly affects older tobacco and alcohol users, in recent years its incidence is also increasing in young people due to changes in sexual habits that predispose to ~~Human Papillomavirus (~~HPV~~)~~ infection, mainly to its 16 genotype at high risk of transformation [2–4].

Although surgical and therapeutic treatments have improved the overall survival (OS) rate of patients (approximately 66% at 5 years) [5], the diagnosis is still late, making it necessary to develop more suitable prevention, diagnosis and treatment measures to better improve detection and life expectancy [6].

Recent growing evidences are increasingly highlighting the pivotal involvement of the microbiota in determining the healthy or disease status of several human districts, such as those mental, respiratory, cutaneous and hepatic [7,8], and that of the vitamin D in several extra-skeletal regions [9–13]. Therefore, research is redirecting its attention towards the deepening of the knowledge on these themes in order to evaluate their reciprocal relation and assess their possible use as potential prognostic biomarkers ~~for prevention, diagnosis and prognosis of many tumor types, including that of the oropharynx~~ [14].

On these bases, this narrative literature review aims to evidence the state of the art regarding oral microbiota and vitamin D contribution in the pathogenesis of ~~oropharyngeal squamous cell carcinomas (~~OPSCC~~),~~ in order to summarize and highlight how vitamin D prevents and opposes infections, and clarify its homeostatic function on the microbiota balance, its cellular effects on human oral cancer cells in *in vitro* studies, and HNSCC patients' clinical features related with its deficiency, to better optimize specific prevention and treatment strategies in the near future.

METHODS

By using the journal citation electronic databases of the MEDLINE U.S. National Library of Medicine (NLM) from PubMed, Scopus, Google Scholar, and the Cochrane Database of Systematic Reviews (CDSR), the following terms “oral microbiota”, “oral virota”, “virus”, “epitheliotropic virus”, “Human Papillomavirus ~~(HPV)~~”, “Epstein-Barr virus (EBV)", “oral bacteriota”, “bacteria”, “Phylum Firmicutes”, “Phylum Fusobacteria”, “Phylum Bacteroidetes”, “*Fusobacterium nucleatum*”, “*Porphyromonas gingivalis*”, “*Streptococcus mitis*”, “*Streptococcus salivarius*”, “oral mycobiota”, “mycetes”, “Candida spp”, “tobacco”, “alcohol”, “head and neck squamous cell carcinoma ~~(HNSCC)~~”,“oral squamous cell carcinoma (OSCC)”, “oropharyngeal squamous cell carcinoma ~~(OPSCC)~~”, “tonsillar squamous cell carcinoma (TSCC)”, “oral cavity squamous cell carcinoma (OCSCC)”, “vitamin D3”, “vitamin D2”, “cholecalciferol”, “ergocalciferol”, “reactive oxygen species (ROS)”, “cytokines”, “IL-6”, “IL-1beta”, “CCL2”, “CXCL2”, “CXCL8”, “CSF3”, “precision medicine”, “immune system”, both in single and/or mutually combined, have been searched, by also using the MeSH vocabulary system, in titles and abstracts in order to find indexed most pertinent articles and reviews; only original researches published in peer-reviewed journals have been considered. The literature search has been limited to the scientific publications in English language of the last 15 years, from the beginning of January 2006 until the end of November 2020, since most of the articles on these topics have had a considerable and exponential increase in this time frame; 620 appropriate abstracts and full papers have been carefully read, screened and reviewed according to the selected and adopted inclusion and exclusion criteria reported in Figure 1; therefore, one hundred and thirty-one have been finally selected, detailed, included and critically commented in the text.

NORMAL ORAL MICROBIOTA

More than 700 bacterial species populate the oral cavity [15], which is ideal for their growth due to its temperature (about 37°C), pH value (between 6.5 and 7.5), and presence of saliva that keeps

them hydrated and fed [15]. Fascinating is that each oral cavity niche is characterized by a peculiar microenvironment [16] that harbours a site-specific microbiota [15]. In particular, the tongue has the highest microbial diversity and contributes to the colonization of the other oral regions [15]. Regarding the salivary microbiota, it is mainly represented by *Streptococcus*, *Prevotella* and *Veillonella* genera, with no gender differences between males and females [16]. Interestingly, a similarity between salivary and oropharynx microbiota has been observed in relation to Firmicutes, Proteobacteria and Bacteroidetes (Figure 2), with each site dominated by distinct families within these phyla [16]. In particular, the prevalent microbes of the oropharynx are *Streptococcus pyogenes*, *S. pneumoniae*, *Haemophilus influenzae* and *H. parainfluenzae* (Figure 2), while *S. faecalis*, *Eikenella corrodens*, Enterobacteriaceae, *Actinomyces*, *Lactobacilli*, *Veillonella* and *Treponema* are dominant in the oral cavity [15]. Yokoyama and coll. have evidenced how bacteria can differ in *in vivo* and *in vitro* conditions, reinforcing the fact that many factors influence their behaviour [17]. Moreover, changes in the local environmental conditions can favour the ~~increase of the disease~~ potential aggressiveness of pathogenic bacteria [15].

Regarding the oral cavity, the bacterial genera present in healthy people are mainly *Actinomyces*, *Capnocytophaga*, *Eikenella*, *Eubacteria*, *Fusobacterium*, *Haemophilus*, *Lactobacillus*, *Leptotrichia*, *Neisseria*, *Porphyromonas*, *Prevotella*, *Propionibacterium*, *Peptostreptococcus*, *Streptococcus*, *Staphylococcus*, *Veillonella* and *Treponema*, with a predominance of communities belonging to Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria phyla [15,18].

This niche is also colonized by commensal epitheliotropic viruses such as HPVs, which have also been detected in gingival biopsies, reservoirs of the virus [19], and by non-pathogenic Candida spp yeast forms [20]. The dynamic balance between the oral microbiota components and the immune system is at the basis of the host oral health; therefore, when a reduction in their number and variety occurs, stronger pathogenic HPVs and *C. albicans* mold forms can become more prevalent and persistent [21,22].

In such a context, the role of probiotics can be determinant for the prevention of such imbalances and crucial for a better prognosis in several diseases~~. For instance~~, since they can enhance the effectiveness of immunotherapies ~~with~~ based on the use of checkpoint inhibitors; as an example, the oral administration of *Bifidobacteria* can control the tumour growth with the same efficiency of PD-L1 specific antibody therapy [23]. Moreover, probiotics can also reduce the mutagenic effects of harmful substances, by modulating the expression of proteins involved in cell proliferation, apoptosis, inflammation or immune system activation [24].

OROPHARYNGEAL SQUAMOUS CELL CARCINOMA ~~(OPSCC)~~

HNSCC accounts for about 5% of all human tumours and represents the sixth most common malignancy worldwide [1,25]. OPSCC, whose annual incidence is estimated to be around 230.000 new cases in the world [26], is the most frequent histological type of HNSCC and originates from the epithelium covering the upper aero-digestive tract, which includes the sino-nasal cavities, the oral cavity, the oropharynx, the hypopharynx and the larynx [27]. ~~***Human Papillomavirus (HPV) infection.***~~ Moreover, it is showing an increased trend over the past 3 decades due to a rising rate in HPV infection [28], with high geographic heterogeneity of positive HNSCC affected cases that goes from about 50% in Europe to more than 70% in North America [29]. These different epidemiological data could be justified by some confounding factors, such as associated smoking and alcohol habits, different sampling methods and HPV infection detection modalities. As well known, the etiological role played by specific high-risk HPV (HR-HPV) genotypes in a subset of OPSCC is now well established [30]. HPV is in fact responsible for the most common sexually transmitted infections and can be detected in oral and oropharyngeal mucosa in about 7% of 14-69 years old people, with a male/female ratio of 3:1. While in most cases mucosal HPV infection self-clearances in 6-24 months, a persistence of HR-HPVs ~~infection (especially of cancerogenic 16 and 18 genotypes),~~ which often occurs with an unbalanced microbiota, can ultimately lead to OPSCC

[31]. ~~On the contrary, low-risk HPV are associated with benign lesions, such as mucosal oropharyngeal papillomas [31].~~

The tonsils and the base of the tongue are the most common involved sites, corresponding to about 46% and 47% of OPSCC, respectively [31]. ~~Patients with HPV-related OPSCC have a younger median age with a white males prevalence and show in most cases non-keratinizing, undifferentiated aspects with basaloid features, high propensity for neck metastases and different biologic behavior [31]. Neck metastases in HPV-positive subjects are characterized by an early onset and peculiar pathological features. Symptoms related to OPSCC are persistent sore throat, dysphagia, sensation of pharyngeal lump, ear pain and painless neck masses that frequently constitute an early sign of disease.~~ HPV-positive OPSCC must be regarded as a distinct molecular and clinical-pathological entity related to HPV infection [28,30,32]; especially HPV16 genotype is estimated to be the main carcinogenic agent in upper airways [33–35]~~. The peculiar anatomical site of cancer onset, rich of lymphoid tissue (i.e. Waldeyer ring), probably explains the different patterns of immune response to tumor cells and the better prognosis of OPSCC. Moreover, HPV-related OPSCC is considered genetically distinct from tobacco-associated carcinomas with differentially expressed genes and lower mutational burden [33].~~ When conditions favour a microbiota depletion, HR-HPVs can become prevalent and inhibit the cellular response to stress, thanks to the action of their viral E6 and E7 oncoproteins, thus leading to DNA-damaged cells uncontrolled proliferation and to a higher risk of cancer onset and progression [21,30,34–36].

While sexual behaviors represent a specific risk factor for increasing HPV-related tumors, smoking and alcohol consumption are well-established conventional risk factors for OPSCC [37]. ~~HPV-positive OPSCC are more common among patients with a lower number of cumulative pack-year tobacco smoking and less alcohol consumption compared to HPV-negative ones [37].~~

~~***Prognostic factors and stratification risk.*** HPV status is considered an independent prognostic factor with better treatment responses, higher OS rates and persistence-free survival rates in HPV-~~

positive compared to HPV-negative OPSCC. However, OS is reduced in HPV-positive smoking patients (considered cut-off < 10 pack-year) [38].

For HPV-related OPSCC, a new classification and a separate staging system have been established in the 8th edition of the American Joint Committee on Cancer-Tumor-Nodes-Metastasis (AJCC-TNM), especially regarding nodal status [39]. The new staging system has been introduced in order to improve treatment strategies, especially for HPV-related OPSCC. According to observed survival data, OPSCC patients are categorized in three prognostic groups: low, intermediate and high risk of death in relation to risk factors (HPV status, smoking and alcohol history, tumor and nodal stages) [38]. Three-years survival rates range from 46 to 93% [30]. From a clinical point of view, HPV-related OPSCC are often diagnosed in early-moderate tumor stages of the disease, according to the new classification system, but with a high nodal spread. Nodal metastases frequently show cystic features at computed tomography scan and nuclear magnetic resonance imaging [30].

HPV-positive OPSCC have distinct risk factors profiles and oncological outcomes compared to HPV-negative cases. Favorable prognostic factors related to HPV infection are younger age, better performance status and smaller primary tumors [30].The well-recognized intrinsic high radio-sensitivity of HPV-related OPSCC could be explained by specific molecular features such as the activation of wild-type p53, the downregulation of cyclin D1, the lack of EGFR amplification and differences in the tumor microenvironment (TME) [30,40–42]. A different tumor-infiltrating lymphocytes pattern with better recurrence-free survival has been described in HPV-positive OPSCC [32]. A high CD8+ T cell infiltration and an increased PD-1 expression are associated with improved survival rates in HPV-related tumors. The favorable outcomes of patients with HPV-related OPSCC can be explained by a stronger immune response against these tumors [43]. A better knowledge of the interaction between HPV and the host's immune system could improve treatment strategies with tailored oncological protocols [44]. Taken as a whole, HPV-related OPSCC distinguish for better oncological outcomes regardless of treatment strategy, since the 5-year

survival rates are of 75-80% and 45-50% in HPV-positive and HPV-negative tumors, respectively [38].

More recently, human microbiota perturbations, which also contribute to HPV infection and persistence [21], have earned a position of primary importance in several cancer types and also in immune and oral disorders. In the very last years, the first steps are moving towards knowledge of their involvement in the genesis and progression of OPSCC and new literature data are evidencing that during OPSCC development important dysbiosis occur [45]. To this regard, a peculiar role for vitamin D also in extra-skeletal regions has been suggested, even if its antimicrobial and antitumoral actions, well-described for the gut, have not been fully documented for the oropharynx yet [46–48].

ORAL MICROBIOTA AND OPSCC

As outlined, the balance in terms of number and variety of commensal and pathogenic bacterial strains is one of the factors that can significantly contribute to oral cancer development and progression. Some pathogens are involved in chronic inflammation through metabolic activities that lead to the production of sulphur compounds, acids and free radicals, thus inducing pro-tumorigenic damages [15,24]. Besides these, several other substances and mechanisms are involved in the initiation and progression of the oncogenic process: in fact, bacterial endotoxins, metabolic by-products and increased enzymatic activities can lead to somatic mutations and signalling pathway alterations [49]. Moreover, inflammatory cells or cytokines, released in the tumour microenvironment (TME) in response to bacterial unbalance, can lead to the production of radical oxygen and nitrogen species, ending in DNA alterations [49]. Additionally, as observed in 2018 by Yost *et al*. assessed that the oral squamous cell carcinoma (OSCC)-associated microbiota secretome is enriched with pro-inflammatory molecules such as LPS, flagella and peptidases, while pro-DNA repair factors are absent [50]. In line with these findings, Hooper *et al*., via a Fluorescent *In Situ* Hybridization (FISH) analysis and a 16S rRNA sequencing of OSCC samples surface, revealed that

their microbiota was mainly composed of *Clavibacter michiganensis, Fusobacterium naviforme, Ralstonia insidiosa* and *Prevotella* spp. According to the authors opinion, the bacteria selection was driven by an acidic and hypoxic microenvironment. However, ~~it remains unclear if this~~ they didn't clarify if this latter selection was a consequence or a leading factor for tumour development [51]. To aggravate the situation, some bacterial species such as *Streptococcus salivarius*, *S. intermedius*, *S. mitis* and non-pathogenic *Neisseria* subspecies, other than Candida spp., produce alcohol dehydrogenase, which, as a consequence of ~~due to~~ ethanol metabolism, is responsible for the production of carcinogenic molecules, such as acetaldehyde (ACH), hydroxyl ethyl- and hydroxyl-radicals~~; these species among them are included~~ (Figure 2) [24,52]. More over~~In fact~~, *Porphyromonas gingivalis*, *Fusobacterium nucleatum, Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* generate volatile sulphur compounds, such as the genotoxic and mutagenic agents hydrogen sulphide and methyl mercaptan, that induce chronic inflammation, cell proliferation, migration, invasion and tumour angiogenesis [15].

By focusing on the microbial communities that have been related to oral cancer, a very huge amount of papers and research approaches have been reported, thus revealing a quite complex, variegated and sometimes controversial scenario. As an example, the pilot study presented by Wolf *et al*., who compared the microbial species present in the saliva of oral cavity (OP)- and OP-SCC affected patients with those of healthy subjects, evidenced through a sequencing analysis, that an early high prevalence of Firmicutes was present ~~has been observed earlier~~ in tumour patients respect to the healthy group, although the importance of the result obtained must strongly be resized due to the limited sample size [14]. The review of La Rosa and colleagues identified, this time in a slightly larger number of cases, a panel of bacteria, including *Capnocytophaga*, *Corynebacterium*, *Haemophilus*, *Oribacterium*, *Paludibacter*, *Porphyromonas* and *Rothia*, to discern ~~oral cavity (~~OC-~~)~~/OP-SCC affected patients and healthy controls; in particular, *Capnocytophaga gingivalis*, *Peptostreptococcus* spp., *P. gingivalis*, *Prevotella* spp., and *Streptococcus* spp. were ~~oral microorganisms~~ the most~~ly~~ associated with saliva samples from OSCC [24]. ~~In another report~~

~~*Streptococcus mutans, Lactobacillus fermentum, L. salivarius* and *L. rhamnosus* have been described to be higher in OPSCC patients (Figure 2) [15]. More in general, Guerrero-Preston's group found that the presence of *Lactobacillus* or the loss of *Haemophilus*, *Neisseria*, *Gemellaceae* or *Aggregatibacter* in saliva could be considered as a HNSCC biomarker; this is the first time that an association between *Lactobacillus*, tumour samples and advanced TNM stage has been evidenced~~. Moreover, by comparing the saliva microbiome of OPSCC and OCSCC patients with healthy controls, they showed that the relative abundance within the genera *Streptococcus*, *Dialister*, and *Veillonella* can be useful to discriminate tumoral from control samples; in addiction, cancer samples lost *Neisseria*, *Aggregatibacter* (Proteobacteria), *Haemophilus* (Firmicutes) and *Leptotrichia* (Fusobacteria) [49]. In another study, the same authors determined a decrease in *Streptococcus* and an increase in *L. salivarius*, *L. fermentum*, *L. gasseri/johnsonii* and *L. vaginalis* (Figure 2) with the progression of the TNM stage [53]. These authors are the few ones who reported observable oral microbiota differences potentially useful as oral cancer biomarkers. Yang *et al*. also showed how bacterial communities dynamically change during OCSCC progression; 5 major phyla differed among healthy and OSCC groups. Firmicutes were the dominant phylum in oral rinse samples (58.40% in healthy individuals, 59.65% OSCC stage 1, 59.76% OSCC stage 2 and 3, 58.43% OSCC stage 4) with a relative abundance of 25% in tumour lesions and 35% in the saliva of OSCC patients. ~~Other differences are that~~ Moreover, stage 4 OSCC showed significantly more oral Fusobacteria than healthy individuals [54], as however also observed by La Rosa *et al*. ~~also found that Fusobacteria are significantly higher in OCSCC~~ [24].

Therefore, considering that each individual possesses his own characteristic oral microbiota ~~and based on the approach of Zhang *et al*., that found significant microbiota differences between cancer sites and normal tissues [55]~~, it will be likely possible to identify new markers for personalised treatment targets [55]. In particular~~fact~~, specific bacterial taxa, such as *Veillonella*, *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Actinomyces*, *Clostridium*, *Haemophilus*, *Enterobacteriaceae* and

Streptococcus spp. ~~are~~ seem to be strongly related to oral cancer and epithelial precancerous lesions [56].

~~Oral microbiota and OPSCC risk factors~~. If on one side microbial communities can be altered by several factors such as age, pH, oxygen levels, nutrients, lifestyle as dietary habits, oral hygiene, tooth loss, periodontal disease, tobacco, alcohol consumption and HPV status [15,57,58], on the other hand the microbiota imbalance itself may facilitate HR-HPV infection and persistence [58].

These significant microbiota differences have been mainly detected in advanced stages of OPSCCs [59]. As an example, Guerrero-Preston and coworkers observed a significant presence of *Gemellaceae* and *Leuconostoc* in HPV-positive compared to HPV-negative HNSCC cases [49] ~~and Banerjee and collaborators identified a specific microbial signature within OCSCC and OPSCC, using a pan-pathogen assay [60]~~. Interestingly, Lim *et al.* proposed a microbiota panel as a biomarker to predict OCSCC and OPSCC (in HPV-positive and -negative subsets) in a clinical setting. The authors observed that, based on the oral microbiota composition, it is possible to discriminate cancer patients from healthy subjects, with reported sensibility and specificity of 100% for OCSSC and 90% for OPSCC [61]. In HPV-positive oral cancer patients, members of *Actinomyces*, *Granulicatella*, *Oribacterium* and *Campylobacter* genera, as well as *Veillonella dispar*, *Rothia mucilaginosa* and *Haemophilus parainfluenzae* significantly increased, while *Streptococcus anginosus*, *Peptoniphilus* and *Mycoplasma* significantly decreased [57].

~~The virota has been also recently studied in early stages of tonsillar cancers (TSCC) and neck metastases by Carey and coll. [62].~~ Conversely, *Lactobacillus*, *Bifidobacterium*, *Atopobium*, *Prevotella*, *Streptococcus* and *Veillonella* genera increased, while *Rothia*, *Neisseria* and *Lautropia* significantly decreased [57]. The oral microbiota composition of smokers and non-smokers was analysed by Rodriguez-Rabassa and coll. in saliva samples [63]. Five phyla resulted most abundant in smokers: Proteobacteria (40%), Firmicutes (29%), Bacteroidetes (23%), Fusobacteria (5%) and Actinobacteria (2%), representing the 99% of the sequences found. In non-smokers, the most represented were Firmicutes (66%), followed by Bacteroidetes (16%), Actinobacteria (5%),

Fusobacteria (5%) and Proteobacteria (4%), representing the 96% of all sequences. At a genus level, *Streptococcus* resulted the most abundant in both groups (15% in smokers and 35% in non-smokers) [63]. The authors also examined the expression patterns of pro- and anti-inflammatory cytokines~~,~~ finding how IL-2, IL-4 and adrenocorticotropic hormone were significantly higher in smokers' samples, while macrophage-derived chemokine, IL-5, IL-7, IL-10, insulin and leptin were down-modulated compared to non-smokers [63]. In another work, a higher abundance of *F. nucleatum* was detected in smokers [15]. Conversely, Fan and co-workers determined how, in the American population, the overall oral cavity microbiota composition differs based on alcohol consumption. In fact, a decreased abundance in Lactobacillales is associated to alcohol consumption and, thus, to a reduced capacity to metabolize ACH to less toxic forms. Alcohol impairs neutrophils function contributing to bacterial overgrowth, increased permeability, microbes penetration, and inflammatory cytokine production from monocytes, thus allowing microbial proliferation [64]. The assessment of the alterations in the oral microbial communities, at the time of diagnosis and during oncological treatments, may be therefore associated to oral tumor risk factors and therefore it was expected to be useful as a prognostic and surveillance biomarker [65].

HPV16 persistence has been often revealed in cancers of the tongue of non-smokers, non-drinkers OPSCC affected patients [16]. ~~Since it is the prevalent genotype in 98% of the OCSCC/OPSCC [60], it could be used as a potential diagnostic and prognostic biomarker~~. Other HPV, such as genotypes 1, 2, 6b, 18, 26, and 34, have been less detected [60].

Epstein-Barr Virus (EBV) has been also retrieved in OCSCC [60]. Despite Broccolo *et al.* evidenced a HPV16/EBV coinfection in about 15–20% of HNSCC in the Italian population, HPV16 appears to play a role mostly in OPSCC, while EBV in OCSCC [66]. HR-HPV oncoproteins can in fact initiate and/or amplify epithelial-mesenchymal transition (EMT) [67], the hallmark of cancer progression and metastasis [68], by cooperating with EBV (Figure 3). Regarding this last virus, its latent membrane protein 1 (LMP1) promotes cell growth, protects cells from apoptosis, enhances cell mobility, stimulates angiogenesis and matrix metalloproteinase (MMP)-9 expression and

downregulates E-cadherin expression, while LMP2A and EBNA-1 increase cells invasive/migration ability [68]. Differently from HPV-positive OPSCC affected patients, EBV status seems to show a statistically significant negative impact on their prognosis [69]. In fact, despite it generally causes benign lesions, it may be involved in lymphomas and malignancies of several different human sites, such as the oral niche. Thanks to its latency, persistence properties and ability to target B cells and keratinocytes of the head and neck region [70], it may immortalize them, thus acting as a tumour progression co-factor rather than a cancer initiator [71].

PERIODONTAL PATHOGENS AND ORAL CANCER

By considering oral cancer affected patients with a history of periodontal disease, a significant increase in the genera *Fusobacterium*, *Eikenella* and *Capnocytophaga* and in the Leptotrichiaceae family has been detected [57]. In particular, *P. gingivalis* and *F. nucleatum* are noteworthy able to induce inflammatory cytokines production, cell proliferation, invasion, migration and inhibition of apoptosis, by causing host cell genomic alterations; moreover, they are both involved in chronic periodontitis which, in turn, correlates with malignant tumour development [15,56].

P. gingivalis is one of the Bacteroidetes phylum members able to convert ethanol into ACH and induce DNA damage, mutagenesis and epithelium hyperproliferation [72]. An association among increased risk of oro-digestive cancer mortality, severity of periodontal disease and its serum IgG levels has been found (Figure 3) by Ahn *et al*. [73]. *P. gingivalis*, together with *F. nucleatum* and the oral carcinogen 4-nitroquinoline-1-oxide, allows the transformation of OSCC and increases the signalling along the TLR2-IL-6-STAT3 axis [72,74–76]. In other experiments, human immortalized oral epithelial cells, persistently exposed to *P. gingivalis* at low multiplicity of infection, showed morphological changes, increased proliferation, migration and invasion [72]. In support of this evidence, several authors suggested that chronic periodontitis is one of the main factors that contributes to the metastatic progression of ~~oral cancer in~~ many oral cancer cells. Cho *et al*. ~~since they~~ observed that *P. gingivalis*-infected YD10B OSCC cells had an increased invasiveness and

EMT–like changes [77]. Other authors demonstrated that prolonged and repeated infection with *P. gingivalis* (twice a week for 5 weeks) enhanced the invasiveness of Ca9-22 OSCC cells through the acquisition of cancer stemness and EMT characteristics, while the short term infection determined morphologic changes (loss of adhesiveness and polygonal shape) in YD10B cells, expression of cancer stemness markers (CD44 and CD133) and EMT [77–79]. In YD10B cells, *P. gingivalis* also demonstrated to increase the expression of MMPs, main effectors of neighbouring tissues invasion [77,80] and to lead ~~It leads~~ to cytokines production, in particular of IL-8, by the epithelial cells, contributing to the inflammatory response (Figure 3) [72,77]. *P. gingivalis* stimulates ZEB1 expression, that influences multiple stages of carcinogenesis, ~~including~~ such as the initial transformation, progression and EMT, thus leading to metastasis and resistance to therapy [81]. It upregulates the expression of B7-H1 and B7-DC on human cancer cells, favouring the production of IL-1, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) [15,72]. B7-H1 can also interact with PD-1 receptors on tumour infiltrating lymphocytes, by blocking their cytotoxic activity against the cancerous epithelial cells [72]. Park *et al.* showed how IgG against *P. gingivalis* and lower serum IL-6 levels positively correlate with the 5-years OS in OSCC patients, thus they might be accurate diagnostic/prognostic biomarkers for OSCC [82].

F. nucleatum triggers reactive oxygen species (ROS) generation leading to NADPH oxidase activation, in particular the NOX1 and NOX2 isoforms; interesting, IL-6, IL-8 and SOD2 gene expression increases in gingival fibroblasts after *F. nucleatum* infection [83]. *F. nucleatum* can induce cellular DNA damages, indicated by up-regulation of the DNA damage sensor histone variant γH2A.X [84].

In their meta-analysis, Bronzato *et al*. highlighted that *Fusobacterium* has 2.93-fold higher chance to be present in tumour lesions and a 6% higher abundance in HNSCC compared to non-tumoral areas. They assessed that it promotes OSCC cells proliferation and disrupts adherence junctions on human tongue dysplasia cells; in addition, it can favour *C. albicans* and *P. gingivalis* colonization (Figure 3) [85]. Recently and for the first time, *F. nucleatum* and *P. aeruginosa* have been assessed

as the 1st and 2nd prevalent microorganisms in HNSCC, respectively (Figure 3) [85]. Since in oesophageal cancer tissues *F. nucleatum* has been associated with a shorter survival time, it has the potential to become a prognostic biomarker [86]. *F. nucleatum* is abundant in OSCC patients; MMP-9 and MMP-13, produced after its infection, together with IL-1a, IL-1b, IL-6, IL-8 through the NF-kB pathway, have been used in monitoring and detecting a metastatic phenotype (Figure 3) [4,77,88].

P. aeruginosa triggers DNA breaks in epithelial cells, thus causing chromosomal instability; this Gram-negative Proteobacteria member possesses LPS, flagella and exotoxin U, with potent pro-inflammatory activities, that, like for *F. nucleatum*, result in neutrophils recruitment through NF-kB signalling pathway. It is also able to disrupt adherent junctions (Figure 3), even if its role in initiation and/or progression of OSCC has not actually been proved [87].

Candida spp. Subjects with *Candida* infection have a two-fold increased risk of developing cancer in mouth, tongue, oropharynx and oesophagus (Figure 3). Candida species are prevalent in oral cancer immunocompromised patients, due to the underlying disease and treatments [16,88]. Candida spp. cause systemic infections in about 74% of OSCC affected patients; the most frequent is *C. albicans* (84%), followed by non-*albicans* strains (23%) [88,89]. In particular, *C. glabrata* metabolizes ethanol to ACH, while other non-*albicans* strains degrade junctional and basement membrane proteins, such as fibronectin and claudins [88], with a proteolytic activity higher than that of *C. albicans* [88]. To this regard, fibronectin and claudins can be important predictive biomarkers for both metastatization and recurrence; in particular, CLDN4 is a potential marker for predicting the outcome of OSCC affected patients [88].

Subjects with higher microbial load and lower salivary flow had more Candida spp. growth, but without any association with a lower OS [89]. Moreover, healthy smokers possess a higher number of Candida spp. compared to non-smokers (28.2% and 13.3%, respectively) [89].

Human beta-defensin-2 (hBD-2) has a potent antimicrobial activity against *C. albicans* and its highest expression has been detected in lung, trachea and tonsils; *in vivo* it is also expressed in

TSCC and in oral epithelial cells [90]. *C. albicans* induces hBD-2 mRNA expression in a dose- and time-dependent manner; but its expression is lower in TSCC and OPSCC than in hyperplastic and healthy tonsils [90]. In the study from Bertolini *et al*., it has been shown that immunosuppression coupled with *C. albicans* colonization results in a bacterial dysbiosis which, in turn, promotes the fungal virulence [22]. In particular, *Enterococcus*, in a rate below 20% in healthy adults, increases up to 82% in chemotherapy-treated patients or with systemic disease. It has also been assessed that the immunosuppression type influences the state of dysbiosis associated with oral candidiasis [22].

PROINFLAMMATORY CYTOKINES AND OPSCC

A gene expression profile, conducted in three different OSCC cell lines by Rao *et al*., showed an up-regulation of genes involved in proliferation and angiogenesis, and a down-regulation of those ones involved in apoptosis regulation, tumour inhibition and keratinisation respect to human oral normal keratinocytes cells; also cytokines, such as IL-8, VEGF, EGFR, STAT CXCL10, CCL5, TGFB2, TNFSF10, as well as VEGF, are 4-fold up-regulated, suggesting that inflammation may play important roles in OSCC [91]. Regarding IL-10, its mRNA expression levels may also independently predict the survival and relapse rates of HPV-positive OSCC patients, thus emphasising its crucial role in the tumoral progression [92]. For IL-8, recognised as an autocrine regulator of OSCC growth and a cell mobility enhancer, an increase expression of its high and low affinity receptor CXCR1 and CXCR2 in oral cancer has been observed; therefore, this salivary cytokine has been proposed to be a discriminative biomarker for oral cancer [80].

VITAMIN D

Vitamin D is a liposoluble steroid hormone, well known for its beneficial role in bone metabolism, calcium/phosphorus homeostasis maintenance and immune function [93]. Its antioxidant effect has been also investigated [94]. The vitamin D2 isoform (ergocalciferol) is produced in plants and yeasts and can be absorbed from the diet or introduced by supplementation; conversely, vitamin D3

(cholecalciferol) can be endogenously synthesized from 7-dehydrocholesterol in sunlight-exposed skin. Vitamin D2 and D3 are inactive pro-hormones, which require a two-step hydroxylation to be converted into a fully active vitamin D form. They are transformed in the liver into 25- hydroxy vitamin D (25[OH]D), which is further activated in the kidney to 1α,25 dihydroxy vitamin D ($1,25[OH]_2D$, calcitriol). Calcitriol has high affinity for the vitamin D receptor (VDR), a nuclear steroid hormone receptor that regulates a variety of genes [95,96]. Bound to its receptor, vitamin D translocates into the nucleus, where, together with the nuclear accessory factor retinoid X receptor (RXR), binds to vitamin D response elements (VDREs) on DNA, resulting in a direct gene transcription activation [97,98].

VDR is also located in the cytosol, modulating vitamin D action via non-genomic mechanisms characterized by rapid activation of intracellular signaling molecules, including kinases, lipases, second messengers and Ca^{2+} and Cl^- channels, with antiproliferative properties and by inducing apoptosis without gene transcription changes [93,99]. ~~The VDR is also present in cancer cells, where it modulates target genes involved in cellular growth, differentiation and apoptosis [100], suggesting a pivotal role of vitamin D in cancer growth and progression [101,102].~~

~~In fact vitamin D, which is downregulated in tumor tissues, generally prevents cancer incidence and progression through the inhibition of cell proliferation, angiogenesis, metastasis and the induction of apoptosis and differentiation [17,18,19].~~

VITAMIN D AND OPSCC

Meta-analyses of observational studies have shown a positive association between low blood 25[OH]D levels and less survival of patients with cancer in several body sites such as colorectal, lung, breast, prostate, head and neck, esophageal, pancreatic, kidney, ovarian and hematologic ones [103].

The main vitamin D effects, that can be involved in the prevention of cancer incidence and progression, include the inhibition of cell proliferation, angiogenesis and metastasis and the

induction of apoptosis and differentiation [17,18,23]. A possible role for vitamin D in preventing cancer growth and progression is also suggested by the presence of VDR in cancer cells, in which it can modulate target genes involved in cellular growth, differentiation and apoptosis [100–102].

Regarding the possible association between vitamin D and HNSCC/OPSCC development, only few studies have been developed. According to the *in vitro* studies analyzed, vitamin D3 and 13-cis retinoic acid have been shown to have equipotent antiproliferative effects on tongue squamous cell carcinoma (SCC-25) cells [104]; 1,25$[OH]_2$D has been shown to inhibit the OPSCC growth by upregulating the cell cycle inhibitor p18 expression [105]; moreover, several tumoral cells produce 1,25$[OH]_2$D to regulate their own growth [106]. To this regard, it has been recently demonstrated that OPSCC cells express high levels of cytochrome P450 2R1 (CYP2R1), which is involved in the conversion of inactive into active vitamin D [107]. Moreover, polymorphisms in CYP27B1 and CYP24A1 genes, other cytochrome P450 family members involved in vitamin D metabolism pathway, seem to affect susceptibility to OPSCC [108], while single nucleotide polymorphisms in the VDR gene have been associated to increase the risk of OPSCC [109].

With respect to the vitamin D effect on cancer patients, recently, the vitamin D status, intake, and metabolism have been considered associated with their outcome [47]. It has in fact been demonstrated that HNSCC patients with lower levels of vitamin D intake are at higher risk of recurrence, suggesting that vitamin D supplementation may be an appropriate intervention for recurrences prevention [46,110] and for OS improvement [103,110,111]. In addition, vitamin D supplementation in esophageal cancer patients has been associated with a longer disease-free survival [112], while in HNSCC patients with a higher or adequate pre-diagnostic plasma vitamin D concentration, it has been reported a notable risk decrease and an improved OS [113]. All these studies suggest a possible prognostic role for vitamin D in the HNSCC context.

Despite the known relation between human papillomavirus (HPV) and fundamental micronutrients, that one made by vitamin D to protect from viral infections, especially in oral cancer has not been clarified yet. A first study, conducted by Özgü E. *et al*, evidenced the inverse relation between

serum 25[OH]D levels and the cervicovaginal HPV infection in sexually active women., thus suggesting that low vitamin D levels could be one of the reasons for HPV persistence [114].

Additionally, vitamin D may also be inversely associated with lymphatic metastasis and a negative HPV status, already known to be a negative prognostic factor [46]. Moreover, the same authors have shown that a severe vitamin D deficiency alters intra- and peri-tumoral immune cell infiltrate levels, while vitamin D administration trigger the cytotoxic activity of patient's NK cells [46].

1,25[OH]$_2$D also modulates the levels of several cytokines in the plasma from patients with HNSCC [115]; this suggests a role for vitamin D in the immune system modulation, also supported by the evidence that it can favor antitumoral immune responses if used as adjuvant of immune therapies based on cetuximab and nivolumab [46].

Considering that other authors suggest that a clinically relevant protective effect of 25[OH]D on oral and OPSCC risk is unlikely and supplementation of the general population with 25[OH]D is not beneficial in preventing these cancer types [116], further research is needed to elucidate the potential effect of vitamin D on OPSCC progression.

VITAMIN D, MICROBIOTA, PERIODONTAL PATHOGENS AND PROBIOTICS

Although a pivotal role for vitamin D in the intestinal homeostasis is well established, less is known regarding its importance in the oral compartment. The intestinal effect is exerted v*ia* many regulatory activities such as calcium and phosphate absorption, protection against infection, anti-inflammatory action and modulation of the gut microbiota [48]. In fact, vitamin D ensures appropriate levels of antimicrobial peptides in the intestinal lumen [48] and the maintenance of the epithelial integrity by modulating the intracellular tight junctions (TJ), real barriers against toxins and enteric pathogens [117,118]. The importance of vitamin D/VDR signaling in intestinal homeostasis is also evidenced during the development of a chronic inflammatory state, when this signaling system is disrupted [119].

Vitamin D can directly control the immune system *via* the VDR in activated or naïve CD4 and CD8 T cells, B cells, neutrophils, macrophages and dendritic cells [117]. In particular, CD4 positive IL-17 or IL-10 producing T cells are common in the gut, where their balance is essential to maintain tolerance and immunity to the resident microbiota [120]. The 1,25$[OH]_2$D_VDR_RXR complex downregulates IL-17 and -23 production, promotes IL-10 production in human B cells and increases chemotaxis and phagocytosis in the innate immune cells [117]. The microbiota also stimulates the maturation and differentiation of T and B cells and promotes IL-10-producing B-cells [121,122]. Therefore, vitamin D and gut microbiota are interdependent, since they control together the immune response of gut and intestinal eubiosis [123]. Interestingly, it has been reported that the expression and activity of VDR is under the control of short-chain fatty acids such as sodium butyrate produced by microbiota [124]. Butyrate has potent health-promoting effects, which results from the fermentation process of indigestible polysaccharides (fibers) from colon microbiota [125].

Overall, these evidences show the strong connection between vitamin D, immune system and gut eubiosis. Since an oral-gut microbiota axis does exist, as has been confirmed by several authors [126], and since vitamin D downregulates NF-kB signaling and proinflammatory cytokines production, protects TJ and inhibits MMPs in OSCC [127], it is reasonable to assume that it could also indirectly preserve oral eubiosis (Figure 3), as indeed pointed out by Robles-Vera *et al*. at least in rats [128].

Not much is known on the specific antimicrobial pathogens mechanisms of vitamin D, although some evidences are pointing out a possible indirect role in infection prevention, mainly due to its immune system regulatory capabilities. This is the case of the recent discoveries made by De Filippis *et al.* that have shown the growth- and adhesion-inhibitory effects of vitamin D towards oral pathogens such as *P. gingivalis*. This hormone inhibited human gingival epithelial (HGE) and periodontal ligament (HPL) cells infection through the modulation of hBD-3 and the reduction of TNF-α, IL-8 and IL-12 production [129].

In a study from Nouari *et al.*, authors have shown that the bioactive vitamin D3 isoform increases M1 monocytes-derived macrophage polarization and their protective phagocytotic and bactericidal activity towards *P. aeruginosa,* by exerting strong immunotherapeutic properties [130]. The antimicrobial properties of vitamin D, also due to its liposolubility, have been also demonstrated against *C. albicans,* without the severe side effects which are conversely exerted by Amphotericin B, the gold standard antifungal treatment [131].

Finally, in an *in vitro F. nucleatum*-driven colorectal carcinoma mice model, vitamin D supplementation has demonstrated to reduce cancer incidence [132]. In fact, the gastrointestinal niche is one of the most important target organs of vitamin D, as demonstrated by the local synthesis of 1,25[OH]$_2$D and VDR expression in most gut cell types [48]. Moreover, subjects with higher 25[OH]D concentration has reduced relative amount of Firmicutes phylum and Clostridia class [133]. Finally, the oral vitamin D supplementation in healthy volunteers has decreased the relative amount of *Escherichia*, *Shigella* spp., *Helicobacter* spp. and *Pseudomonas* spp. [134].

While several studies have shown that certain pathogens downregulate VDR expression, while others can also cause its increase in the colon [135], probiotic treatment with *L. plantarum* and *L. rhamnosus* enhances the levels of VDR protein in human and mouse intestinal epithelial cells, and prevents Salmonella-induced colitis in wild-type mice, but not in VDR -/- mice [136].

In a multicentric study, double-blind, placebo-controlled, randomized, oral supplementation with probiotic *L. reuteri* NCIMB 30242 has improved circulating 25[OH]D levels relative to placebo [137].

All these evidences suggest that the role exerted by vitamin D on the oral cavity is most probably due to an indirect effect mediated by the immune system stimulation. Considering the positive effects of probiotics on vitamin D circulating levels, the supplementation of specific probiotic strains together with vitamin D may be valuable in deficient HNSCC affected patients.

CONCLUSION

Overall, the different experimental study models found in the literature ~~have~~ evidenced the key role of some microorganisms in oropharyngeal tumorigenesis: both *P. gingivalis* and *F. nucleatum* induce an inflammatory state, together with *P. aeruginosa*, HPV-16 and Candida spp., reinforcing their link to several diseases of the oral niche.

An important role for vitamin D is beginning to be glimpsed also in this specific context. In fact, oral pathogens presence seems to be mediated by extra-skeletal vitamin D effects, with eubiosis as a prerequisite for well-being, and dysbiosis as an antechamber for carcinogenesis. Nevertheless, up to now, while a direct vitamin D antimicrobial protective role in gut health has been already confirmed, it has not been fully recognized for the oropharynx yet.

Further and deeper functional characterization studies and investigations of the mechanisms and factors that condition microbial diversity in the oral niche are therefore required to *i)* fully understand how single or combined oral microbiota shifting and vitamin D levels influence cancer development and *ii)* better define the risk factors and the tumoral biomarkers useful to establish specific OPSCC prevention strategies and optimize clinical practice.

ABBREVIATIONS

ACH, Acetaldehyde; EBV, Epstein-Barr Virus; EMT, Epithelial-Mesenchymal Transition; ~~FISH, Fluorescent *In Situ* Hybridization;~~ HNSCC, Head and Neck Squamous Cell Carcinoma; HPV, Human Papillomavirus; HR-HPV, High-Risk HPV; IL, Interleukin; MMP, Matrix

Metalloproteinase; OCSCC, Oral Cavity SCC; OPSCC, Oropharyngeal SCC; OS, Overall Survival; ROS, Reactive Oxygen Species; RXR, Retinoid X Receptor; TJ, Tight Junctions; TME, Tumor Microenvironment; TNF-α, Tumour Necrosis Factor Alpha; TSCC, Tonsillar SCC; VDR, Vitamin D Receptor.

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Figure legends

Figure 1. Flow-chart of the criteria adopted for the narrative literature review.

Figure 2. Healthy and tumoral oropharyngeal microenvironment.

Figure 3. Microbial effects on oral epithelial cells and possible vitamin D-mediated oropharyngeal cancer protection mechanisms.

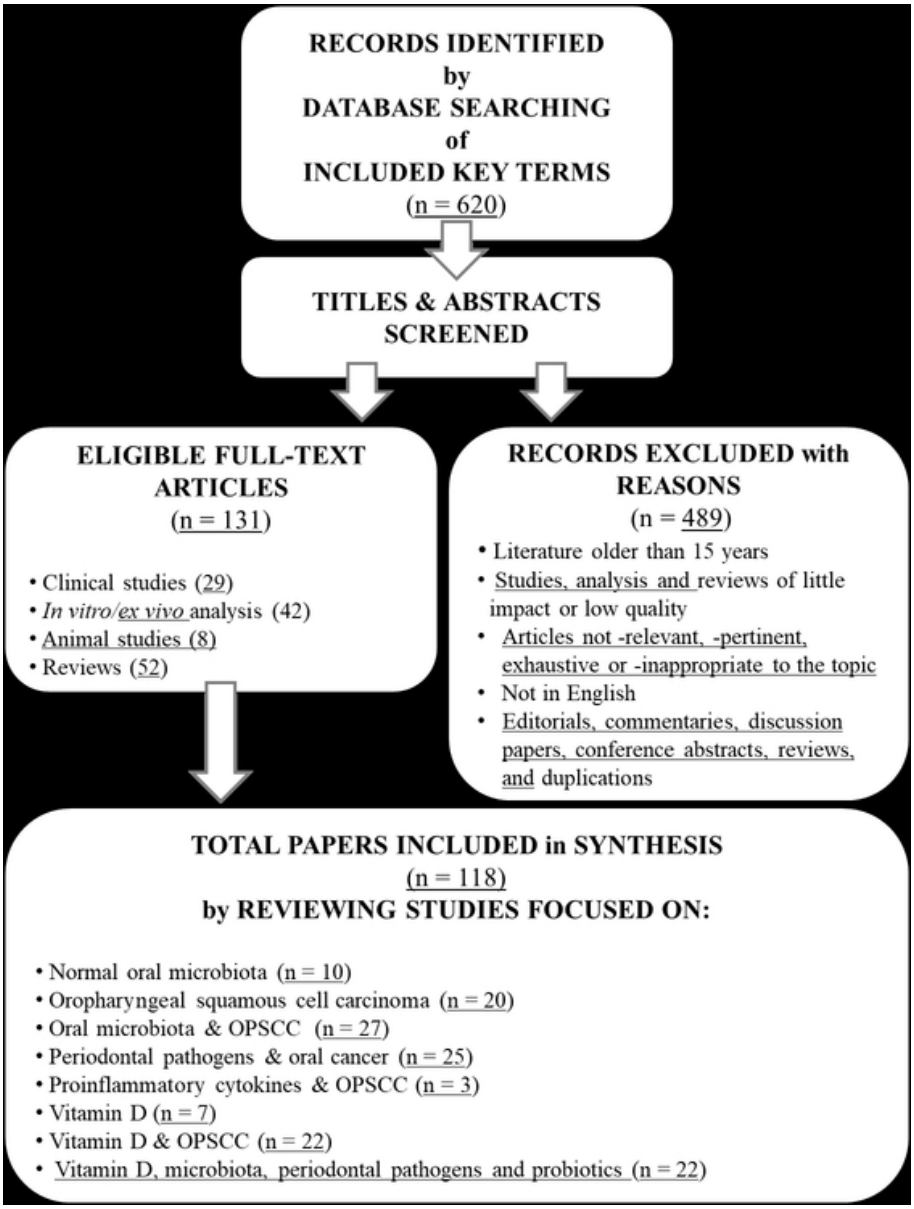

Figure 1. Flow-chart of the criteria adopted for the narrative literature review.

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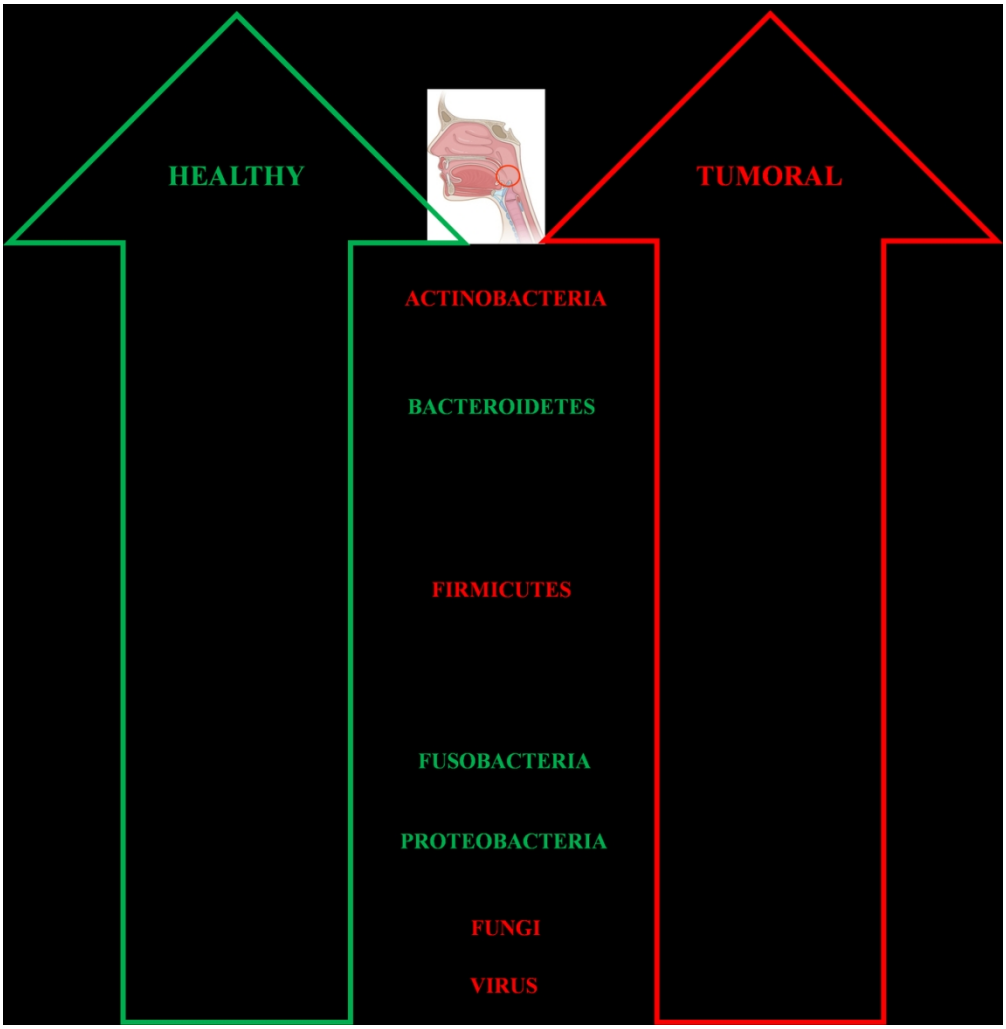

Figure 2. Healthy and tumoral oropharyngeal microenvironment.

113x115mm (300 x 300 DPI)

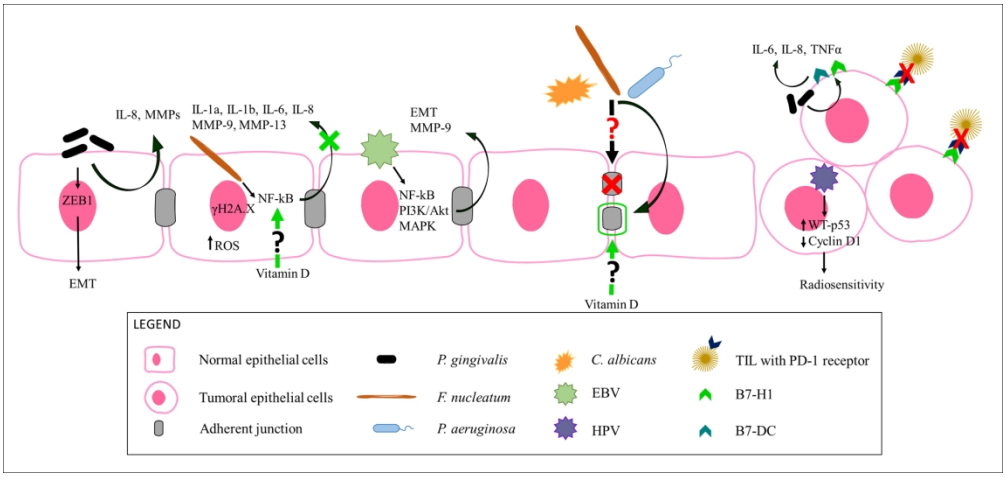

Figure 3. Microbial effects on oral epithelial cells and possible vitamin D-mediated oropharyngeal cancer protection mechanisms.

87x40mm (600 x 600 DPI)