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Oropharyngeal Squamous Cell Carcinoma: Human Papilloma Virus Coinfection with *Streptococcus anginosus*

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Introduction: Human papilloma virus (HPV) and oral bacteria capable of acetaldehyde production from ethanol, such as *Streptococcus anginosus*, *Prevotella melaninogenica*, and *Fusobacterium naviforme* are among oropharyngeal squamous cell carcinoma (OSCC) infectious risk factors. Objective: Determine associations with HPV and *S. anginosus, P. melaninogenica*, and *F. naviforme* in patients with and without OSCC. Methods: Presence of HPV and HPV-16 was determined in 26 patients with OSCC and 26 without OSCC by conventional PCR and simultaneous presence of *S. anginosus, P. melaninogenica*, and *F. naviforme* quantification through q-PCR. Statistical analysis was carried out using Pearson's X^2 and Student's-t test. Results: Patients with OSCC had HPV and HPV-16 frequencies of 84% and 61.5%, respectively, in contrast for patients without OSCC frequencies were 34.6 and 30.7%. *P. melaninogenica*, and *F. naviforme* microorganisms were not present in any participant in this study. *S. anginosus* frequency in patients with OSC was 38.4% and in patients without OSCC was 30.7%. Patients with OSCC had *S. anginosus* + HPV co-infection at a 38.4% frequency and *S. anginosus* + HPV-16 at a 23.1% frequency. For individuals without OSCC *S. anginosus* + HPV co-infection was 3.8% and *S. anginosus* + HPV-16 3.8%. A greater frequency of *S. anginosus* + HPV co-infection and *S. anginosus* + HPV-16 was observed in patients with OSCC in comparison with individuals without OSCC, suggesting the importance of detecting HPV/HPV-16 and *S. anginosus* simultaneously in individuals at risk of developing OSCC.

Key Words: human papilloma virus, bacteria, oropharyngeal squamous cell carcinoma, streptococcus.

Introduction

Carcinomas correspond to 90% malignant tumors in the oropharynx (1). Excessive alcohol and tobacco consumption or Human Papilloma Virus (HPV) infection are main risk factors associated with Oropharyngeal Squamous Cell Carcinoma (OSCC) (2). As evidenced in OSCC patient's epidemiological studies prognosis can vary, depending on risk factors associated with this tumor. As a case in point, patients with OSCC and alcohol and tobacco consumption present poor prognosis, high aggressiveness and a survival rate below 5 years. In contrast, non-alcohol and tobacco consumers with HPV positive tumors present a better prognosis, low resistance to radiotherapy and even survival rate close to 5 years. Some explanations suggest this difference is due to the high number of carcinogens present in tobacco and alcohol metabolites, increasing the number of genetic alterations, whereas patients suffering only from HPV don't present them (3,4).

HPV induced carcinogenic process has been widely studied in cervical cancer with extrapolation to OSCC, based on epithelial similarities. Cell processes such as proliferation, immortalization and cell transformation are promoted by E6 and E7 HPV viral genes, degrading p53 protein and sequestering pRb respectively, acting as cell cycle checkpoint regulators from G1 to S phase progress (5). In Colombia the most common viral types associated with OSCC are HPV-16 and -58 and cervical cancer HPV-16, 18, 45, 33, 31, 52, 58 and 35 (6).

Additionally, Silins et al. (7) described in a cervical cancer model that *Chlamydia trachomatis* infection increased HPV infection and persistence. Bacterial infection provokes epithelial disruptions and micro-abrasions, facilitating HPV entry (8), due to host reaction against the virus, altering the immune response (9). Findings reported in the literature lead us to propose the hypothesis that oral bacterial infections can facilitate HPV infection and persistence, thus promoting OSCC carcinogenic processes.

Some authors have observed presence of *Streptococcus anginosus* is tightly associated with the oropharyngeal carcinogenic process, evidenced by increase in acetaldehyde concentration, a carcinogenic molecule, resulting from alcohol metabolism produced by bacteria in the oral cavity (10,11). Furthermore, Sasaki et al. (12) reported *S. anginosus* antigen (SAA) culture supernate production, a

bioactive antigen, which induced nitric oxide up-regulation in tumor tissues. Moreover, it has also been described that up-regulation of both nitric oxide synthetase (NOS) and cyclooxygenase-2 (COX-2) could be associated with a risk of cancer (13,14). In addition, accumulated data have revealed a significant correlation between *S. anginosus* and carcinogenesis in the upper aerodigestive tract (15,16), mainly due to infection with this microorganism results in increased pro-inflammatory cytokine production in esophageal cancer cell lines (17). Moreover, authors, such as Hooper and collaborators described *Prevotella melaninogenica* and *Fusobacterium naviforme* bacteria can also be associated with carcinogenic processes, as they are also present in patients with oral cancer and absent in patients without cancer (18).

Sufficient evidence indicates Human Papilloma Virus (HPV) is a risk factor associated with oropharyngeal squamous cell carcinoma (OSCC), mainly in the tonsils, altering cellular processes, such as proliferation and cell death, leading to keratinocyte transformation. However, suffering from HPV solely is not sufficient to cause oropharyngeal cancer, since other adjuvant risk factors can be present, including tobacco consumption, alcohol intake and even co-infection with other oral bacteria. In this study presence of HPV and HPV-16 was compared with co-existence and quantification of *P. melaninogenica, F. naviforme* and *S. anginosus* in patients with and without OSCC. At present there are few studies in oropharyngeal cancer, where co-infection with HPV and bacteria, including *S. anginosus* have been performed (19). Taking into consideration, leading investigations in this regard have been made in the cervical cancer model and its association with HPV and *C. trachomatis*.

Material and Methods

Study Population

This work was a multicentric, descriptive cross-sectional study, carried-out in the cities of Bogotá and Manizales, Colombia between 2014 and 2017. 26 patients were eligible for inclusion, as they were diagnosed with oropharynx cancer and confirmed by histopathological findings as OSCC, with indication to be submitted to surgery. After surgery all patients were followed-up for 60 months for survival. Additionally, a control group consisting of 26 individuals with similar ages and sex as OSCC patients were included. Control group individuals did not present apparent lesions in the oral cavity after clinical inspection by their dentist. An oral mucosa sample was collected from control patients by trained personnel with a cytobrush. Samples were obtained after signed informed consent and an epidemiological survey was completed. Protocol for this study was approved by the Pontificia Universidad Javeriana Dental Research Ethics Committee.

DNA Extraction and Quality Evaluation

OSCC patient tumor tissue was divided in two, one fragment was used for diagnosis confirmation by histopathological analysis and the other was placed in RNALatter®. Cytobrush collected from control individuals was also placed in RNALatter®. DNA extraction was performed using the AllPrep Quiagen® kit following manufacturer's instructions. Obtained DNA was quantified using Nanodrop 1000® ranging from 25 ng/µL to 75 ng/µL. Quality of all collected DNA samples was confirmed by amplification of human β-globin gene by conventional PCR using PCO3 forward primer 5' ACACAACTGTGTTCACTAGC3' and PCO4 reverse primer 5' CAACTTCATCCACCTTCACC3', amplifying a 110 bp product. Each reaction contained 1X de buffer, 3 mM MgCl2, 300 nM each primer forward and reverse, Taq polymerase 1U and 200 µM each dNTP, brought to a final volume of 25 µL. Amplification was carried-out in MyCyclerTM (BioRad Laboratories, USA) thermocycler under the following conditions: one 3-min cycle at 95 °C followed by 40 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. Last, an extension final phase was included at 72 °C for 5 min. PCR products were analyzed by electrophoresis in 2% agarose gel stained with 1 X SYBR safe®. As a positive control 25 ng lymphocyte DNA was used and as negative control PCR mix. Bacterial DNA present in samples was evaluated by real-time PCR with amplification of 16S gene. Reaction mix consisted of 1 X BioRad® Master mix, 300 nM primers (forward) 5´TCCTACGGGAAACCGGGGCAGCAGTA3´ and (reverse) 5´GGACTACCAGGGTATCTAATCCTGTTAAT3´ and 200 nM probe FAM-CGTATTACCGCGGCTGCTGGCATTCGC– BHQ1, and 1 µL DNA. Amplification was carried-out in CFX96 de BioRad® performing an initial denaturing phase at 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s and an annealing phase at 62 °C for 45 s, where fluorescence was read. As a positive control *Porphyromonas gingivalis* DNA was used and as a negative control mix without DNA.

HPV detection: Positive DNA for β-globin and 16S rRNA amplification was processed for generic HPV amplification. To this end MY09 primers (forward) 5´CGTCCMARRGGAWACTGATC3´ and MY11 (reverse) 5´GCMCAGGGWCATAAYAATGG3´ were used. Reaction mix consisted of 1 X de buffer, 3 mM MgCl2, 200 nM dNTPs, 300 µM primers (each forward and reverse) and 1U Taq polymerase. Amplification polymerase consisted of one 95°C cycle for 5 min, followed by 40 cycles of 95 °C during 1 min, 55 °C during 1 min and 72 °C during 1.5 min. Last, a final extension phase at 72 °C for 5 min was carried-out. PCR products were visualized in 2% agarose stained with 1X SYBR safe®, amplified product was 450 bp. As positive

controls DNA from SiHa and HeLa cell lines were used, and as negative control PCR mix without DNA. Samples positive for HPV were again amplified to detect HPV-16 serotype specifically, as this is the most common viral genotype present in oropharyngeal cancer. Primers used were forward primer: 5′ AGCAGAACCGGACAGAGCCCA3′ and reverse primer 5′ TCTGAGAACAGATGGGGCACACA3', amplifying the E7 region of the HPV-16. Reaction mix contained 1X buffer, 3 mM MgCl2, 200 nM dNTPs, 300 µM primers (each forward and reverse) and 1U Taq polymerase. Amplification protocol consisted of one 95 °C cycle for 5 min followed by 62 °C for 1 minute and 72 °C for 1 minute and a final extension cycle at 72 °C for 5 min. PCR products were visualized in 2% agarose gel stained with 1X SYBERsafe®. Expected product was 158 bp long. As a positive control DNA from SiHa cell line was used and as a negative control PCR mix.

Microorganisms of interest identification: For *P. melaninogenica*, *F. naviforme* and *S. anginosus* detection and quantification a construct was designed that contained analyzed bacteria sequences (Table 1). Based on the methodology described by Montalvo et al. (20) from construct design ten base dilutions were performed from 1 x 10^6 copies to one copy in triplicates. Reaction mix contained 1 X BioRad® master mix, 300 nM primers (each forward and reverse), 200 nM hybridization probes and 1 µL DNA PCR product. Amplification protocol was performed in CFX96 de BioRad® staring with a denaturing phase at 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, and an annealing phase at 60 °C for 45 s, where fluorescence reading was performed. As a negative control reaction mix without DNA was included. Absolut quantification was performed using construct standard curve from 1 x 10^6 copies to one copy, and slope was calculated and R^2 for each bacterium (Fig. 1). Multiple PCR sensibility for each bacterium was 100 copies.

Result Analysis

Statistical analysis was descriptive with absolute and relative frequencies and central tendency measurements. Comparisons between OSCC patients and individuals without cancer with respect to categorical variables were analyzed by non-parametric Pearson's X^2 test. To compare number of copies for each bacterium in both study groups an unpaired Student's -t test was performed. Last, to evaluate association between HPV, co-infection and the three bacteria in both study groups a non-parametric X^2 was carried-out and patient survival analysis was performed by Kaplan-Meier curve and Log-Rank test. All analyzes were

Table 1. *Streptococcus anginosus, Prevotella melaninogenica* and *Fusobacterium naviforme* primer sequences and probe sequences. Forward and reverse primer sequences and hybridization probe sequences for studied bacteria

Bacteria	5′to 3' primer sequence and probe
Streptococcus anginosus	forward CTAATACATGCAAGTAGGACGCAC reverse CAAGCATCTAACATGTGTTACATACT probe ACCTGCCTATTAGAGGGGGA
Prevotella melaninogenica	forward CAAGTCTCTGTTCCGCTACC reverse ATCAAGCCCCTAAGTACCGT probe AGTCGGGGTGTTCCTTCTTT
Fusobacterium naviforme	forward GCGATCCGGAGCGAATCTAA reverse CAGCTCCCTCCCTAAGGTTG probe TACGTTCCCGGGTCTTGTAC

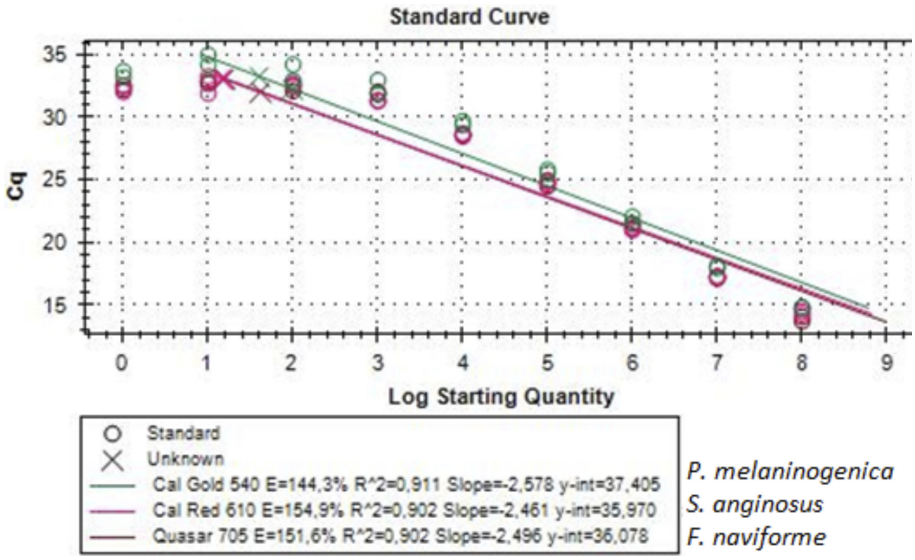

Figure 1. *S. anginosus, P. melaninogenica* and *F. naviforme* quantification. Bacteria quantification standard curve. Construct copy number from one million copies to one copy.

executed using SPSS software. Statistically significance was determined with a $p<0.05$.

Results

Sociodemographic Characteristics

As much as 65.4% of patients with OSCC and 61.5% of patients without OSCC were male (Table 2). Regarding their age, 80.7% of patients with OSCCs and 84.5% of patients without OSCCs were older than 50 years old. With respect to the number of sexual partners the greatest frequency was observed for patients with OSCC (42.4%), whereas for patients without OSCCs it was of 34.6%, affirming having between 1 to 2 sexual partners in all of their life. Regarding risk factors, such as alcohol and tobacco consumption it was found 73.1% (19/26) of OSCCs patients did not smoke and 53.8% did not consume alcohol. Individuals without oropharyngeal cancer 77% (20/26) did not consume tobacco and 50% did not consume alcohol (Table 2). Respecting their socioeconomic levels 34.6% of the patients with OSCC belonged to a low-income level, and a similar percentage to a medium socioeconomic level, whereas for individuals without oropharyngeal cancer, 65.4% pertained to a medium socio-economical class.

Table 2. Sociodemographic characteristics for the studied population

Characteristic	Cases *n* (%)	Controls *n* (%)	p value [a]
TOTAL	26 (50)	26 (50)	
Sex			0.7734
Male	17 (65.4)	16 (61.5)	
Female	9 (34.6)	10 (38.5)	
Age			0.5734
31-40 years old	1 (3.9)	1 (3.9)	
41-50 years old	4 (15.4)	3 (11.6)	
51-60 years old	4 (15.4)	9 (34.6)	
61-70 years old	8 (30.7)	8 (30.7)	
>70 years old	9 (34.6)	5 (19.2)	
Sexual partners			0.6560
0	0 (0)	3 (11.6)	
1 to 2	11 (42.4)	9 (34.6)	
3 to 4	6 (23)	6 (23)	
5 to 6	1 (3.9)	2 (7.7)	
7 or more	6 (23)	2 (7.7)	
NR [b]	2 (7.7)	4 (15.4)	
Smoking			0.7488
Yes	7 (26.9)	6 (23)	
No	19 (73.1)	20 (77)	
Alcohol consumption			0.7814
Yes	12 (46.2)	13 (50)	
No	14 (53.8)	13 (50)	
Socioeconomic status [c]			0.3065
Low	9 (34.6)	9 (34.6)	
Medium	9 (34.6)	17 (65.4)	
High	4 (15.4)	0 (0)	
NR [b]	4 (15.4)	0 (0)	

[a] Chi-square test. [b] No response. [c] Based on criteria by the Colombian Secretary of District Planning and the National Administrative Department of Statistics (Departamento Administrativo Nacional de Estadística - DANE, Decree 304, 2008).

HPV detection and HPV-16 genotype

All participants in this study (n=52) were screened for HPV through conventional PCR, using MY9/MY11 generic marker. Low, high and undetermined risk HPV was detected in 88.4% (23/26) participants of all 26 patients with OSCC. HPV frequency obtained in participants without OSCC was 34.6% (9/26). According to previous findings, HPV-16 is the most frequent HPV genotype in this type of tumor, therefore in the present study this type was detected in a specific manner, amplifying the E7 region of this virus. For the OSCC group 61.5% (16/26) were positive for HPV-16 and for individuals without oropharyngeal cancer 30.7% (8/26) were positive for this viral type (Fig. 2).

P. melaninogenica, F. naviforme and S. anginosus Frequency of Infection

None of the 52 participants presented *P. melaninogenica* or *F. naviforme* infection. However, *S. anginosus* was present in 38.4% OSCC patients (10/26) and 30.7% without oropharyngeal cancer (8/26).

Following, *S. anginosus* was quantified for each study group by q-PCR absolute quantification, and the number of *S. anginosus* copies for each study group was observed (Fig. 3). Even though no significant differences were detected between groups p=0.4637, a slight increase in the quantity of this bacteria in OSCC patients was identified in comparison to individuals without oropharyngeal cancer.

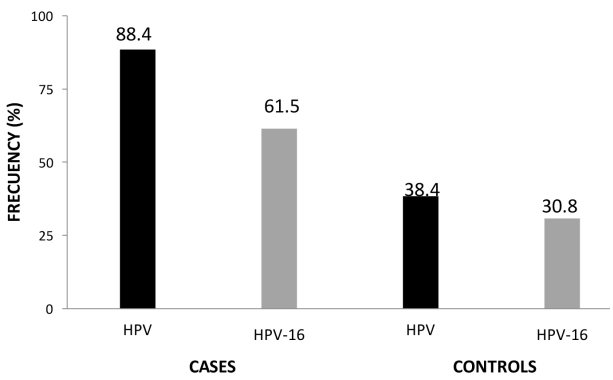

Figure 2. Frequency of HPV detection and HPV-16 in patients with OSCC and non-cancer controls.

It is worthy to point out, of the 10 patients with OSCC and positive for *S. anginosus*, only one case (10%) presented less than 10,000 copies, three cases (30%) less than 100,000 copies and six cases (60%) more than 100,000 copies; whereas eight individuals without OSCC were positive for *S. anginosus*, three (37.5%) presented less than 10,000 copies, another three (37.5%) less than 100,000 copies and only two (25%) presented more than 100,000 copies.

Co-Infection Entre HPV and S. anginosus

Furthermore, to establish in both study groups the possible co-infection association between *S. anginosus* presence or absence and HPV or HPV-16 infection an analysis was performed. 43.4% (10/23) OSCC patients were infected with *S. anginosus* and HPV. Additionally, 37.5% (6/16) presented infection with *S. anginosus* and HPV-16. For participants without oropharyngeal cancer it was observed out of the eight infected with *S. anginosus*, only one presented co-infection with HPV and HPV-16. As determined by X^2 test no significant differences (p=0.761) were observed when OSCC patients (*S. anginosus* + HPV and *S. anginosus* + HPV-16) were compared to control group. Alcohol consumption precedence and *S. anginosus* infection or *S. anginosus*-HPV or *S. anginosus*-HPV-16 co-infection are presented in table 3. In addition to alcohol consumption antecedent for OSCC patients it was observed 60% presented *S. anginosus* infection and 50% presented *S. anginosus* and HPV-16 infection. For individuals without orophanrygeal cancer and alcohol consumption precedence, 62.5% had *S. anginosus* infections and all (100%) presented *S. anginosus* and HPV-16 co-infection.

Last, OSCC patient survival was analyzed with a Kaplan-Meier curve (Fig. 4), comparing only HPV positive OSCC patients with and those co-infected with HPV and *S. anginosus*. No significant differences were observed as demonstrated by Log-Rank test (p=0.559). Nevertheless, a tendency towards less survival probability was recognized for patients co-infected with both microorganims.

Discussion

HPV in OSCC gains each day more attention due to increased incidence in youngsters without alcohol and tobacco history, mainly associated with sexual practices namely oral sex (4). The present study revealed a 76.9% HPV frequency rate in patients with OSCC, in agreement with a report by Steinau et al. (21) in the USA, where out

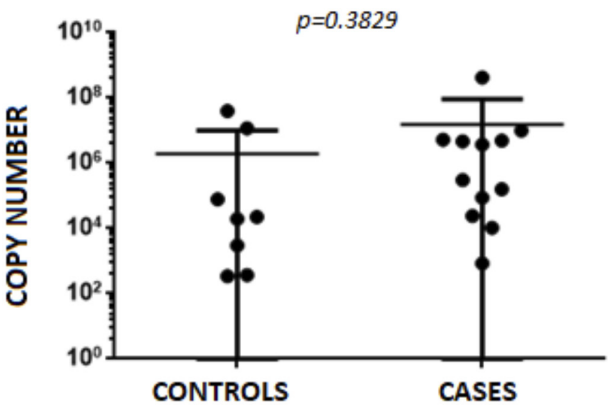

Figure 3. *S. anginosus* copy number in patients with OSCC and non-cancer controls.

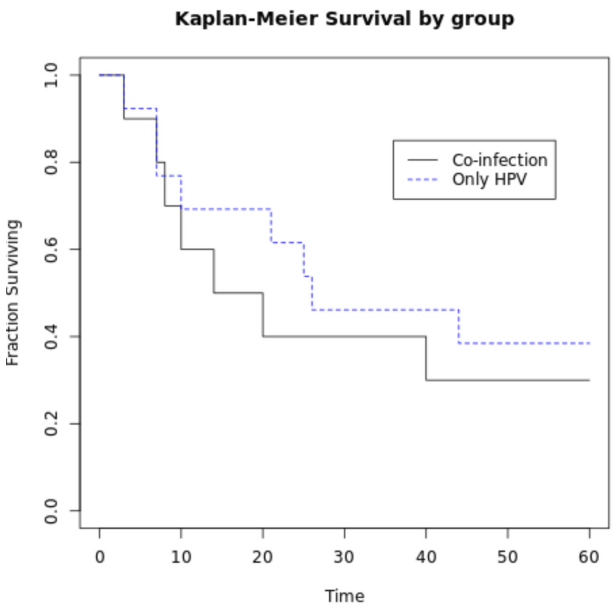

Figure 4. Kaplan-Meier survival curve comparing patients who were only positive for HPV infection (blue dashed line) and patients positive for HPV and *S. anginosus* (co-infection).

Table 3. *S. anginosus* + HPV and *S. anginosus* + HPV-16 association and history of alcohol consumption

Characteristic	Cases n (%)		Controls n (%)	
	Alcohol consumption	No alcohol consumption	Alcohol consumption	No alcohol consumption
S. anginosus	6 (60.0)	4 (40.0)	5 (62.5)	3 (37.5)
S. anginosus + HPV	5 (62.5)	3 (37.5)	1 (50.0)	1 (50.0)
S. anginosus + HPV-16	3 (50.0)	3 (50.0)	1 (100.0)	0 (0.0)

of 557 patients with OSCC 72% were positive for HPV. In Colombia, Erira et al (22) found lower frequencies of 21.74%. Differences between both studies could be accounted by designed primers used for gene amplification and sensibility analysis techniques employed. In the present study conventional PCR was performed using MY9/MY11 primers, which can detect more than 45 viral types, while in other studies (6,22) study Luminex system was used with BSGP5+/BSGP6+ primers detecting 24 viral types.

In regard to sociodemographic characteristics for the OSCC group, as well as individuals without oropharynx cancer, no significant differences were observed. According to what was predicted for both groups in this study they presented similarities in all evaluated sociodemographic aspects. It is important to note a greater number of males (65.4%) in comparison with women (34.6%) had OSCC, in agreement with reports in the literature, where it is suggested male OSCC incidence is greater than female (23). Additionally, most OSCC patients were older than 50 years old (80.7%) and did not present previous history of alcohol consumption (53.8%) or smoked cigarettes (73.1%) similar to what was reported by Bernal (24).

For none of the groups analyzed in the present study presence of *P. melaninogenica* and *F. naviforme* was observed. In contrast, Egwari et al. (25) described a 3.2% frequency in odontogenic tumors. Golin et al. (26) reported 84.5% bacteria frequency of *Bacteroides sp, P. melaninogenica*, *Fusobacterium sp, Veillonella sp, Peptostreptococcus sp, Propionibacterium sp, Bifidobacterium sp* and *Clostridium sp* in oropharynges' from alcoholic individuals compared with 30.5% frequency in non-alcoholic individuals. Mager et al. (18) compared saliva microbiota in 45 patients with oral carcinoma and 229 healthy individuals and quantified 40 bacteria of oral importance in these two analysis groups. An elevated *P. melaninogenica*, *Capnocytophaga gingivalis* and *S. mitis* count was observed in patients with oral carcinoma in comparison with healthy individuals ($p<0.001$). Their results differ from *P. melaninogenica* findings in the present study, possibly explained by the origin of collected samples. Mager et al. (18) carried out their study with saliva samples; in contrast this study used tumor samples or swabs, taking into account the oral cavity provides different niches with unique conditions for presence of certain bacteria populations. As a case in point, Perera et al. (27) gathered up to date information related to oral cancer, describing *Fusobacterium* and *Porphyromonas* are common in tissues and swabs of patients with cancer, whereas *Lactobacillus*, *Rothia* and *Gemella* are frequent in saliva. *Streptococcus* genus is frequently found in patients' tissues as well as in saliva. It is important to note the viridans group of the *Streptococcus genus* is usually present in oropharynx and gastrointestinal tract and genitals (28). Not too long ago it was associated with normal flora (29), however, this affirmation has been changing based on findings mainly focused on *S. anginosus*, where it has been implicated in upper digestive tract cancer (30). For example, *S. anginosus* can induce an elevated acetaldehyde concentration production from ethanol in alcoholic beverages, a metabolite considered carcinogenic (31). Moreover, *S. anginosus* presents in addition to acid production a higher tolerance to this low pH, facilitating oral cavity and gastrointestinal tract infection. Additionally, this microorganism induces expression of inducible nitric oxide synthase and pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6. Therefore, excessive growth of this bacterium could lead to chronic inflammation and increased risk to develop cancer (30).

Recently, a study performed by Wang et al. (32) evaluated specific *Streptococcus* lymphocyte T cytotoxic response in patients with oral cancer at initial and advanced stages and non-cancer controls. They found increased LT CD8+ in patients with cancer in comparison with control group. When analyzing in depth different *Streptococcus* species they found significant increase in lymphocyte T granzyme B expression specific for *S. anginosus*, from early oral cancer stages to advanced stages in cancer patients compared with non-cancer control group. Moreover, this microorganism has been proposed as a biomarker to distinguish between gastric cancer and superficial gastritis, in addition to serve as an indicator of gastric cancer progress (33).

With reference to *S. anginosus* frequency, in the present study we highlight these bacteria were detected in 38.5% of OSCC patients. Similar results have been reported by Sasaki et al. (29), describing a 45.2% frequency for *S. anginosus* in patients with OSCC. Guerrero-Del-Cueto et al. (34) evaluated 43 patients with hematologic and solid cancer finding a 32.6% frequency. Morita et al. (35) reported 44% frequency in esophageal cancer and 13% in oral cancer. Shiga et al. (15) and Tateda et al. (36) reported *S. anginosus* DNA sequence was found in 100% DNA samples obtained from head and neck cancers.

In addition to *S. anginosus* detection in the present study is was possible to quantify this bacterium through qPCR, finding higher levels of this bacterium in OSCC patients compared with non-cancer oropharynx controls. Similar results were reported by Mager et al (18) and Sasaki et al. (29) in the same year, where *S. anginosus* increase was observed in oral cancer patients in comparison with healthy individuals.

Another study performed in healthy individuals by Yokoyama et al. (37) evaluated acetaldehyde production from two saliva bacterial communities. The first was characterized by *S. salivarius* (bacterial species belonging to

the viridans streptococci group) and *Rothia mucilaginosa*. The second community was represented by *Neisseria flavescens* and *Fusobacterium periodonticum* presence. Contrary to what was expected they observed the second community produced less acetaldehyde in comparison with the first community. *In vitro Neisseria flavescens* studies have revealed this species produced the highest acetaldehyde concentrations in comparison with other bacteria, when cultured by itself. This behavior changes if *Neisseria flavescens* is cultured with other bacteria or in community with *F. periodonticum*. Collectively, *in vitro* bacterial characteristics not necessarily reflect their complex habitat behavior, suggesting the importance of evaluating complex interactions present in oral microbiota, resulting from unexpected phenotype expression by the microbial community. Moreover, in the Yokoyama et al. (37) study it was proposed the second community was associated with healthier conditions (less dental caries and non-smokers) in comparison with the first community. These findings are relevant for the present study, since bacteria possibly behave in a distinct manner depending on microbiota found in their natural habitat, suggesting *S. anginosus* phenotype behavior could be modulated by the presence of other microorganisms in the oral cavity, in this case HPV.

Börnigen et al. (19) found bacterial community alterations associated with risk factors in patients with oral and oropharyngeal cancer compared with healthy individuals were studied. In the present study 43 out of 121 patients with oral/oropharyngeal cancer also had an HPV infection; in contrast to the control group, where only one patient out of 242 analyzed was positive for HPV. Microbiota analysis obtained from oral rinse was performed through 16S rRNA sequencing analysis allowing identifying bacterial genera and for a few cases even bacterial species. In addition, the authors described in HPV positive patients with cancer *S. anginosus*, *Peptoniphilus* and *Mycoplasma* were significantly lower. In contrast, *Actinomyces, Granulicatella, Oribacterium* and *Campylobacter* were increased, as well as *Veillonella dispar*, *R. mucilaginosa* and *Haemophilus parainfluenza*e bacteria. However, *S. anginosus* frequency in patients with or without cancer was not specified in the Börnigen et al. (19) study nor how many were co-infected with *S. anginosus* + HPV.

Collectively, in the near future it is necessary to perform further studies with oral pre-neoplastic lesions or include cohort studies to corroborate if HPV interaction with *S. anginosus* is cooperative or occurs at random.

In the present study, it may be concluded that co-infection with *S. anginosus* and HPV or HPV-16 was higher in patients with oropharyngeal cancer compared with non-cancer controls. To date no studies have addressed *S. anginosus* and HPV co-infection frequency in any pathology, including oropharynx cancer. Around 60% of patients presenting *S. anginosus* and HPV co-infections had a history of alcohol consumption, as well as 50% of patients with *S. anginosus* and HPV-16 co-infection had the same precedent. High *S. anginosus* and HPV and *S. anginosus* and HPV-16 co-infection rates in patients with OSCC, indicates the importance of detecting these two microorganisms in individuals with history of alcohol consumption, since evidence suggests a cooperative association between *S. anginosus* and HPV or HPV-16.

Resumo

O vírus do papiloma humano (VPH) e bactérias orais capacidade de produção acetaldeído a partir do etanol, tais como *Streptococcus anginosus*, *Prevotella melaninogenica* e *Fusobacterium naviforme*, estão entre os fatores de risco infecciosos do carcinoma de células escamosas de orofaringe (CCEO). Determinar associações o VPH e *S. anginosus*, *P. melaninogenica*, e *F. naviforme* em pacientes com e sem o CCEO. A presença de VPH e VPH-16 foi determinada em 26 pacientes com CCEO e 26 sem CCEO por PCR convencional e presença simultânea de quantificação de *S. anginosus*, *P. melaninogenica* e *F. naviforme* por meio de q-PCR. Uma análise estatística foi realizada por meio do t-Student e χ^2 de Pearson. Os pacientes com CCEO apresentaram frequências de VPH e VPH-16 de 84% e 61,5%, respectivamente, em contraste, para os pacientes sem CCEO frequências de 34,6 e 30,7%. Os microorganismos *P. melaninogenica* e *F. naviforme* não estavam presentes em nenhum dos participantes deste estudo. A freqüência de *S. anginosus* em pacientes com CCEO foi de 38,4% e em pacientes sem CCEO foi de 30,7%. Os pacientes com CECO apresentaram coinfecção com *S. anginosus* + VPH em uma freqüência de 38,4% e *S. anginosus* + VPH-16 com freqüência de 23,1%. Para os indivíduos sem CCEO, co-infecção com *S. anginosus* + VPH foi de 3,8% e *S. anginosus* + VPH-16 foi de 3,8%. A Maior frequência de co-infecção com *S. anginosus* + VPH e *S. anginosus* + VPH-16 foi observada em pacientes com CCEO em comparação com indivíduos sem CCEO, sugerindo a importância da detecção de VPH / VPH-16 e *S. anginosus* simultaneamente em indivíduos em risco de desenvolver CCEO.

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