Abstract: Our objective was to compare archived tissue biopsy samples from the oral cavity and oropharynx, in terms of human papillomavirus (HPV) 16 infection. We used Taqman real-time PCR assay to detect HPV16 in 121 archived biopsy samples from the oral cavity and 100 samples from the oropharynx. Among patients with oral cavity cancer (OCC), 9% (6/65) had HPV16 infection which was significantly less than those with oropharyngeal cancer (OPC) where 79% (39/50) were HPV16 positive ($P < 0.001$). Our results demonstrated a significant difference between genders where males had a seven times higher chance of having HPV16 infection ($P < 0.001$). Viral load variation between each group was demonstrated. The median viral load in OPC was similar in OCC cases, but cancer samples were significantly higher than in non-cancer cases (oral cavity samples $P = 0.015$; oropharynx samples $P = 0.09$). From our results, we conclude that there is a significant difference in HPV16 detection between OCC and OPC, and HPV16 differs greatly between male and female cancer patients. (J Oral Sci 58, 265-269, 2016)

Keywords: oral cancer; oropharyngeal cancer; HPV; HPV16; oral tissue biopsy; real-time PCR.

Introduction
The oral cavity and oropharynx, which lie in close proximity to each other, seem to differ in terms of HPV16 infection and association with squamous cell carcinoma (SCC) (1,2). The oropharynx includes the base of the tongue, soft palate, tonsils, and tonsillar region, whereas the oral cavity encompasses the remaining internal tissues of the mouth. Plausible reasons for differences between the two is the fact that the oropharynx contains tissue similar to the cervix in that they are both derived from endoderm (3), and the tonsillar crypts potentially act as reservoirs for HPV (4,5). Oropharyngeal cancer (OPC) rates are expected to surpass the number of cervical cancer rates within 5 years while oral cavity cancer (OCC) rates seem to be on the decline (6). The role of HPV16 in the carcinogenesis of OPCs has been exhibited and known for quite some time now (7-9). With the increasing evidence of certain sexual practices being a significant risk factor for oral HPV infection (10-12), HPV vaccination could act as an important primary preventive measure.

We sought to compare HPV16 infection in tissue biopsies taken from oral cavity cancer (OCC) and oropharyngeal cancer (OPC) patients in order to elucidate any differences between the two locations regarding oral HPV infection.

Materials and Methods
Study population
Archived formalin-fixed paraffin embedded (FFPE) tissue blocks, collected between the years of 2000-2014, were obtained from the University of Washington’s Pathology repository. The types of FFPE tissue blocks requested to be cut for sampling were: 56 non-cancer from the oral cavity, 65 OCC, 50 non-cancer from the oropharynx, and 50 OPC. Gender, race/ethnicity, age at time of biopsy, and cancer status of the samples are presented in Table 1. In order to ensure random sampling, we did not request for a specific number of female/male tissue samples. The IRB guidelines of University of Washington were
followed (IRB# 39027, approved September 2014).

Collection and DNA purification
DNA was isolated from 80 µm tissue block sections using RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol.

HPV and analytic methods
PCR is considered to be highly sensitive and is the most established method for HPV detection in oral rinse sample (13,14). Thus, Taqman real-time PCR assays were used for detection on the ABI Prism 7900 Sequence Detection System with 40 cycles in a reaction (denaturation at 95°C, annealing and extension at 60°C). We used primers and probes specific to the HPV16 E7 region in order to detect HPV16 (Table 2). Absolute quantification was used to determine HPV16 viral load, and total human genomic DNA in the sample was determined on Alu sequences. Serial dilutions of human genomic DNA and full length HPV16 plasmids, of known concentrations, were used as standard curves.

Data analysis was completed using Stata MP 13.1 (StataCorp LP, College Station, TX, USA). Chi² or Fisher’s exact, and Mann-Whitney tests were used where appropriate to compare proportions. t-tests were used to compare mean log of HPV16 viral load.

Results

The majority of the oral cavity biopsies with no cancer were men (64%), Caucasian (63%), and had a mean age of 49 (Table 1). Patients with OCC were mostly men (63%), Caucasian (65%), and had a mean age of 59. Of oropharyngeal biopsies with no cancer, the majority were women (66%), Caucasian (66%), with a mean age of 37. Also, 90% of OPC patients were men, Caucasian (78%), and had a mean age of 54.

Of those with cancer, only 9% of the oral cavity biopsies tested positive for HPV16 compared to 79% of oropharynx biopsies ($P < 0.001$), thus demonstrating a statistical significant difference between sites (Table 3).

It was shown that 35% (48/139) of all biopsy samples in males had HPV16 infection compared to only 9% (7/82) of females ($P < 0.001$, data not shown). When comparing HPV16 prevalence within cancer cases, 50% (43/86) of males were positive compared to only 7% (2/29) of women ($P < 0.001$; Table 4).

Observations in HPV16 viral load, among those positive for HPV16, indicated that those with cancer had a higher concentration of HPV16 DNA than those with no cancer (oral cavity samples $P = 0.015$; oropharynx samples $P = 0.09$; Fig. 1).

Discussion

Tobacco use and alcohol consumption are well-established risk factors for OCC (15), however, there has been
an increase in OPC in younger individuals without these typical risk factors (16,17) where HPV infection has been identified as the etiologic agent (1,18,19). It was once thought that tobacco and alcohol-related oral cancers followed a different pathway than HPV-associated oral cancers. However, more recent studies show a connection with smoking and acquiring oral HPV infection (20). In fact, a large cross-sectional study by Fakhry et al. (21) demonstrated oral HPV16 prevalence to be higher in current tobacco users compared with never or former tobacco users (n = 6,887, P = 0.004). Thus, such evidence suggests that smokers are much more susceptible to oral HPV infection, thus increasing their risk for OCC/OPC.

A global systematic review and meta-analysis including 148 studies with 12,163 head and neck squamous cell carcinoma (HNSCC) cases from 44 countries has been published (22). Results indicated that 3,837 cases were positive for HPV DNA; of those positive for HPV16 DNA specifically, 41% (95% CI 34.4-47.0) were from the oropharynx and 15% (95% CI 11.1-19.1) from the oral cavity. The authors also found that HPV DNA prevalence differed by anatomical site and geographical region where OCC had the highest prevalence in Asia and South Central America, and where OPC was higher in North America compared to Asia, Oceania, and Europe (P < 0.0001). This worldwide picture is in concordance with our results as we found almost a 9-fold difference between HPV16 detection in OCC and OPC (Table 3). This statistically significant variance between infection sites warrants further investigation into the mechanisms associated with oncogenesis.

In the US, the incidence of OPC among men was 4-fold higher than women (23), and a meta-analysis by Ndiaye et al. reported an increased but not significant overall HPV DNA prevalence in men. Our study showed a significant gender difference; males were four-times more likely to have HPV16 infection in general, and seven-times more likely within cancer cases (Table 4). HPV vaccination in boys has been approved in Canada, the United States, Australia, South Korea, and the United Kingdom. However, due to the mounting evidence that HPV-associated OPCs and OCCs affect men at significantly higher rates, it is imperative that adolescent boys are indeed being vaccinated in the approved countries, and to have it implemented globally. A fairly recent study demonstrated a 93.3% vaccine efficacy in preventing oral HPVs in 5,840 women in Costa Rica (24). This same protection should occur in males, but further studies are still needed.

| Table 3 HPV16 status in oral cavity and oropharyngeal archived biopsy samples |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Oral cavity | Oropharynx | Total n | n HPV+ | % HPV+ | Total n | n HPV+ | % HPV+ | P value |
| Cancer+ | 65 | 6 | 9% | 50 | 39 | 79% | <0.001 |
| Cancer– | 56 | 9 | 16% | 50 | 1 | 2% | 0.018* |

*Fisher’s exact test

| Table 4 HPV16 prevalence in males and females |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Males | Females | Total n | n HPV+ | % HPV+ | Total n | n HPV+ | % HPV+ | P value |
| Cancer+ | 86 | 43 | 50% | 29 | 2 | 7% | <0.001* |
| Cancer– | 53 | 5 | 9% | 53 | 5 | 9% | 1.00* |

*Fisher’s exact test

Fig. 1 Viral load in HPV16 positive samples. The gray boxes demonstrate range from lower to upper quartiles. The median is represented by a horizontal line. Maximum and minimum values are indicated by the vertical lines. Viral load variation between each group was demonstrated. The median viral load in OPC was similar in OCC cases, but cancer samples were significantly higher than in non-cancer cases (oral cavity samples P = 0.015; oropharynx samples P = 0.09).
HPV viral load seems to play a role when determining the correlation with carcinogenesis. Certain studies present an association with high viral load and high-grade squamous intraepithelial lesions (25), cellular abnormalities (26), clinically relevant cervical lesions (27), and invasive cervical cancer (28). On the other hand, Lorincz et al. assessed whether high viral loads would predict future risk of cervical intraepithelial neoplasia (CIN) 3 or cancer (CIN3+) (29). They found the presence of HPV had an impact on developing CIN3+, but high viral load did not further predict risk of CIN3+. Regarding oral HPV infection, Martin et al. demonstrated HPV16 viral load in cancer samples to be much higher than in non-cancer samples, which agreed with our results shown in Fig. 1 (30). If the malignancy is directly related to HPV infection then this finding is warranted, however further investigation on oral HPV viral load and carcinogenesis is pertinent.

Limitations of our study include not having any history of sexual practices and number of lifetime partners, which would have allowed for more depth within our study; not having a random sampling of archived tissue blocks so as to deter bias; as well, HPV16 detection does not necessarily indicate carcinogenic involvement, proof of viral transcriptional activity would have been beneficial.

From our results, we conclude that there are significant differences between OCC and OPC regarding HPV16 infection. There is a discernable difference between males and females with HPV16 status where males have a higher prevalence altogether. With our conclusions, and the rising amount of evidence that certain sexual practices increase risk of HPV infection, it seems imperative that males should be vaccinated at a young age in synchrony with females.

References