

**The Affect of the Oral Microbiome in Health and Disease on the Uptake of HPV in  
Oral Keratinocyte Cells**

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THESIS

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This thesis is dedicated to my wife, Seema, who provided loving support to me throughout my entire residency program.

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## TABLE OF CONTENTS

<u>CHAPTER</u>	<u>PAGE</u>
I. REVIEW OF RELATED LITERATURE	1
A. Basic Biology of Human Papillomaviruses .....	1
B. HPV Pathology .....	3
C. HPV and the Oral Cavity .....	4
D. HPV and Carcinogenesis .....	6
E. HPV Mechanism of Entry .....	7
F. Detection Methods for HPV .....	11
G. HPV and Association with Microbes .....	16
II. MATERIALS AND METHODS	19
A. Participants .....	19
B. Sample Collection .....	20
C. DNA Purification .....	20
D. Quantitation of 16 sRNA Levels .....	20
E. Conversion of 16 sRNA to Levels of Taxonomic Units .....	21
F. Statistical Analysis of Microbial Population .....	22
G. Trypan Blue Exclusion .....	22
H. Micronucleus Assay .....	22
I. Production of Human Papillomavirus Pseudovirus (PsV) .....	23
J. Pseudovirus Infection .....	23
K. Quantitation of Pseudovirus Entry .....	24
III. RESULTS	25
A. HPV-16 Entry Assays .....	25
B. Micronucleus Assay .....	29
C. HPV-16 Entry in Presence of Tobacco Products .....	30
D. 16 sRNA Microbial Identification .....	32
IV. DISCUSSION	39
V. CONCLUSIONS	42
REFERENCES .....	43
VITA .....	48

## LIST OF FIGURES

<u>Figure</u>		Page
1.	HPV-16 Entry Buccal Mucosa.....	25
2.	HPV-16 Entry Oropharynx.....	26
3.	HPV-16 Entry at Various Oral Sites.....	27
4.	HPV-16 Entry Assessed Through In vitro Assay.....	28
5.	Micronuclei Assay.....	29
6.	HPV-16 Entry in the Presence of Tobacco Products...	30
7.	Derived Carcinogens Affect on HPV-16 Entry.....	31
8.	Oropharynx – <i>Novosphingobium</i> .....	33
9.	Oropharynx – <i>Prevotella</i> .....	34
10.	Oropharynx – <i>Rothia</i> .....	35
11.	Oropharynx – <i>Neisseria</i> .....	36
12.	Oropharynx – <i>Haemophilus</i> .....	37
13.	Oropharynx – <i>Gemellaceae</i> .....	37
14.	Oropharynx – <i>Porphyromonas</i> .....	38

## **LIST OF ABBREVIATIONS**

PV	Papillomavirus
HPV	Human papillomavirus
ICTV	International Committee on the Taxonomy of Viruses
dsDNA	Double stranded deoxyribonucleic acid
LCR	Long control region
E	Early
L	Late
WHO	World Health Organization
FEH	Focal epithelial hyperplasia
SSC	Squamous cell carcinoma
OPSCC	Oropharyngeal squamous cell carcinoma
HNSCC	Head and neck squamous cell carcinoma
TA	Transit amplifying
pRB	Retinoblastoma protein
PML	Promyelocytic
PCR	Polymerase chain reaction
LCM	Laser capture microdissection
FF	Fresh frozen
FFPE	Formalin-fixed paraffin-embedded
HC II	Hybrid capture II
RLU	Relative light units
ISH	In situ hybridization
RT	Reverse transcriptase
IHC	Immunohistochemistry
PAH	Polycyclic aromatic hydrocarbon
HSGPB	Heparan sulfate proteoglycan binding protein
OTU	Operational taxonomic unit
BIOM	Biological observation matrix

## **SUMMARY**

Human papilloma virus oncogenic subtype 16 (HPV 16) entry into oral keratinocytes can result in an enhanced risk for malignant transformation. This occurs at selective sites in the oral cavity such as the oropharynx and less often in other areas such as gingiva, which also can present with HPV infection of oral mucosa. Although epidemiological evidence suggests several factors that may increase risk of HPV-16 positive oropharyngeal squamous cell carcinoma (OPSCC), there is currently no assay that can predict a particular individual's risk for infection with the virus. Currently identification of a current HPV 16 identification is used as an indication for future rates of infection of HPV 16 without a determination immediate entry functionality. The purpose of this study was to assess immediate entry functionality independent of previous HPV 16 infection.

This study suggests that HPV 16 entry may be affected by the oral microbiome and tobacco smoke components in subjects that had no prior history of HPV 16 infection. Microbiome and oral keratinocyte physiology affects HPV 16 entry and requires further study to enhance our understanding of the stratum spinosum's potential role as a reservoir for HPV 16. Tobacco smoke association with enhanced periodontal disease risk also is associated with DNA damage and a generalized change in oral microbiome diversity, number and bacterial genera.

## **I. REVIEW OF RELATED LITERATURE**

### **A. BASIC BIOLOGY OF HUMAN PAPILLOMAVIRUSES**

Papillomaviruses (PVs) have existed and evolved over the course of the last few hundred million years. These viruses infect the epithelium of numerous vertebrate species of birds, reptiles, and mammals causing chronic asymptomatic lesions and neoplasms (Chow, Broker, and Steinberg 2010; Bernard et al. 2010; Doorbar et al. 2015). PVs are non-enveloped and have an icosahedral structure that is approximately 50-60 nm in diameter (Hausen 2002; Doorbar et al. 2012; Doorbar et al. 2015). Today, there have been more than 200 papillomaviruses identified and sequenced, with over 150 human papillomavirus (HPV) genotypes (Chow, Broker, and Steinberg 2010; Doorbar et al. 2015). According to the International Committee on the Taxonomy of Viruses (ICTV), HPVs are found in five different genera (Alpha, Beta, Gamma, Mu, and Nu) within the Papillomaviridae family (Chow, Broker, and Steinberg 2010; Bernard et al. 2010; Doorbar et al. 2015; Doorbar 2006; Doorbar et al. 2012; de Villiers et al. 2004; Kines et al. 2009; Hausen 2002). The genomes of PVs are made of circular double-stranded DNA (dsDNA) and are typically about 8 kb in size (Doorbar 2006; Doorbar et al. 2015; Bernard et al. 2010; de Villiers et al. 2004). Phylogenetically, the various genera within the Papillomaviridae family are at least 10% different than PVs from other genera. The various “types” of PV under a particular species share at least 80-90% of the genome in common (Doorbar 2006; de Villiers et al. 2004; Chow, Broker, and Steinberg 2010).

Understanding the organization of the HPV genome and resultant viral proteins is important when discussing the pathophysiology of an infection. Replication of the genome occurs via multi-copy, extrachromosomal plasmids. The long control region



(LCR) accounts for 400-700 base pairs of the genome, but does not code for any viral proteins. The LCR functions as a binding site for transcription factors and various regulatory proteins (Doorbar 2006; Chow, Broker, and Steinberg 2010). Open reading frames are designated as early (E) or late (L) (Chow and Wang 2010; Chow, Broker, and Steinberg 2010;). Transcription occurs in a 5' – 3' direction (Doorbar 2006; Chow, Broker, and Steinberg 2010). Generally, the E1 and E2 genes play a role in replication, while L1 and L2 are important for packaging of the virus prior to release. There is great variation in the roles of the remaining early genes leading to differences in pathogenicity among different HPV types (Doorbar 2006; Doorbar et al. 2015).

The E1 and E2 proteins mainly support viral DNA replication. E2 binds to sites on the viral and host genome. E1 is a replicative DNA helicase necessary for replication and amplification of the viral genome (Chaudhary et al. 2009; Chow, Broker, and Steinberg 2010). Interestingly, it is the only enzyme encoded by PVs (Allen et al. 2010; Chow, Broker, and Steinberg 2010). The functions of E2 are highly dependent on interactions with the host DNA and can act to regulate E6 and E7. E4 is critical to virus particle release. Along with E5, E4 is very heterogeneous between HPV types, which may account for differences in tissue tropism (Lerma Puertas et al. 2011; Doorbar et al. 2015). The E5 has been shown to prevent apoptosis in cells afflicted with DNA damage. E5, E6 and E7 are all capable of proliferation-stimulating activity (Chaudhary et al. 2009; Hausen 2002). It is well documented that the encoded proteins from E6 and E7 play a leading role in malignant transformation, demonstrating the ability to immortalize various human cell types in culture (Allen et al. 2010; Hausen 2002). The role of E6 and E7 will be more specifically discussed in the section describing the mechanism of HPV infection.

## B. HPV PATHOLOGY

Closely related HPV types can display distinctly different pathologies. Epithelial tropisms determine whether the virus will infect either a mucosal or cutaneous surface. These tropisms are thought to be regulated at the level of viral gene expression and conformational changes of the capsid at the time of infection (Smith et al. 2004; Doorbar et al. 2015; D'Souza et al. 2005; Chuang et al. 2008). Tropism is also likely determined through the chemical interaction with the epithelial target cell through binding to heparan binding proteins and proteoglycans such as syndecans 1,4. Cutaneous manifestations of disease associated with HPVs include common warts, plantar warts, flat warts, filiform warts, pigmented warts, epidermoid cysts, and skin cancer. Condyloma acuminatum, focal epithelial hyperplasia, cervical neoplasms and cancer, head and neck cancers, anogenital cancers are mucosal diseases associated with HPVs (Chuang et al. 2008; Doorbar et al. 2015).

The Alpha, Beta, and Gamma genera of HPVs are larger and much more diverse than the Mu and Nu genera. Historically, the fifteen species from the Alpha-HPV genus have been the most widely studied because they are transmitted sexually and have the ability to cause significant disease. In addition to being classified as mucosal or cutaneous, the Alpha-HPVs are further categorized as high- or low-risk based on molecular biological data. The low-risk mucosal types such as HPV-6 and HPV-11 rarely cause neoplasms. High risk-types of HPV have the ability to immortalize keratinocytes causing pre-malignant and malignant lesions(Chaudhary et al. 2009). The World Health Organization (WHO) has identified twelve HPV types known to be

carcinogenic including HPV-16. Numerous other types from the Alpha genus are considered probable carcinogens but there is a current lack in epidemiologic data to confirm (Chow, Broker, and Steinberg 2010; de Villiers et al. 2004; Doorbar et al. 2015; Doorbar et al. 2012).

### C. HPV AND THE ORAL CAVITY

The prevalence of HPV in the oral cavity and oropharynx has not been extensively studied, but it is estimated that at least 25 types are associated with oral lesions (Castro and Filho 2006). Oral lesions associated with HPV include squamous cell papilloma, condyloma acuminatum, focal epithelial hyperplasia (FEH), verruca vulgaris (common wart), and malignant lesions such as squamous cell carcinoma (SCC)(PhD 2014)

Oral and oropharynx squamous cell papillomas are soft tissue tumors that are most commonly found on the soft palate, tongue, frenii and mandibular labial mucosa(Castro and Filho 2006). Low-risk HPV genotypes 6 and 11 are most commonly associated with these lesions. Papillomas appear as exophytic growths demonstrating a pedunculated base(Castro and Filho 2006). The surface contour and color varies. Papillomas can appear to have small finger-like projections, giving them a verrucous appearance. Color is dependent on the vascularity and level of keratinization.

Condyloma acuminata are typically found on the skin and mucosa of the anogenital area. Oral manifestation is the result of oral sex or self-inoculation. HPV types 6, 11, and 16 are most commonly involved. Lesions often present in clusters and can be found on the lips, tongue, gingiva, and soft palate. The warts are sessile or

pedunculated with a cauliflower-like appearance (Castro and Filho 2006; Prabhu and Wilson 2013; PhD 2014).

Verruca vulgaris, also known as the common wart, can appear in the oral cavity as a result of autoinoculation from a skin lesion. The HPV is often transmitted from skin on the hand or a finger to the labial mucosa. The most common genotypes associated with verruca vulgaris are HPV 2 and 4. The well-circumscribed lesions are sessile and exophytic with marked hyperkeratosis. The oral presentation is clinically and microscopically indistinguishable from skin lesions. Treatment involves surgical excision and recurrence is rare (PhD 2014; Castro and Filho 2006; Prabhu and Wilson 2013).

Oral focal epithelial hyperplasia (FEH) or Heck's disease is a rare condition most commonly seen in children. Both HPV 13 and 32 have been identified as the etiology. FEH can affect many oral surfaces but is usually associated with the buccal or labial mucosa. Clinical presentation includes multiple clustered lesions that are asymptomatic. Coloration typically reflects the normal appearance of the mucosa with little keratinization. Treatment is reserved only for functional or esthetic concerns and includes surgical excision, laser ablation, cryotherapy, and cauterization (Prabhu and Wilson 2013; Castro and Filho 2006; PhD 2014).

#### **D. HPV AND CARCINOGENESIS**

The role of HPV as an etiologic agent for cervical cancers has been well established and documented in the literature for decades. Historically, head and neck squamous cell carcinomas (HNSCCs) have been attributed to long-term exposure to

known carcinogens such as tobacco products(Allen et al. 2010). Over the last couple decades, the etiologic role of high-risk HPVs have been implicated in a large subset of HNSCCs, specifically for oropharyngeal squamous cell carcinoma (OPSCC). A 2012 systematic review and meta-analysis reported a significant increase of HPV prevalence in association with HNSCC. Articles included published prior to 2000 show a prevalence of 40.5%, with an increase to 64.3% reported between 2000 and 2004. Prevalence jumped to 72.2% most recently between 2005 and 2009(Mehanna et al. 2012). A 2011 multi-center, retrospective study of 271 OPSCCs also found that HPV prevalence increased from 16.3% between 1984-1989 to 71.7% between 2000 and 2004(Chaturvedi et al. 2011). Chaturvedi et al. predict that the annual number of HPV-positive OPSCCs will surpass the number of cervical cancer cases by the year 2020. It is also estimated that OPSCCs will represent a majority of all head and neck cancers in less than 20 years(Chaturvedi et al. 2011).

A number of HPV types including 18, 31, 33, and 35 have all been detected in HNSCCs, but HPV 16 is by far the most common(Mehanna et al. 2012; Allen et al. 2010; Chaturvedi et al. 2011). The HPV associated SCCs demonstrate distinct differences in epidemiology, clinical presentation, and molecular biology that will further be discussed in this paper(Doorbar et al. 2015; Allen et al. 2010; Chaturvedi et al. 2011).

Patient survival is perhaps the most significant aspect of HPV-associated OPSCC that differs from HPV-negative cancers(Hennessey, Westra, and Califano 2009). A recent meta-analysis reported an 18% reduction in death among patients with HPV-positive HNSCC compared to HPV-negative patients. Further, the study found that there was also a 38% reduced risk of disease failure in HPV-associated HNSCC patients. When looking

at site-specific results, Ragin et al. found that HPV-positive oropharyngeal tumors had a 28% reduced risk of death and 49% lower risk of disease failure when compared to HPV-negative oropharyngeal tumors. HPV association with non-oropharyngeal tumors was not found to impact risk of death. Patient with larynx tumors were found to have two times greater risk of disease failure when HPV-positive compared to being HPV negative(Allen et al. 2010). These results suggest a HPV 16 induced selection of more differentiated but DNA damaged/mutated target keratinocytes (e.g., stem cell, transmit amplified, TA and clones) with a loss of function of tumor suppressor genes as noted above. This is expected to result in a depressed gatekeeper genes effect such p53 and Rb with predominance by oncogenes such as c-MYC in a large compartment (population of cells) from basal to suprabasal regions to enhance growth of a malignant transformed state. Response to treatment and improved survival over HPV 16 negative related oropharynx carcinomas is also expected to be a product of restraint on caretaker gene damage and a low level of nuclear instability(Frank 2003).

#### E. **HPV MECHANISM OF ENTRY**

The natural host for completion of the HPV lifecycle is the squamous epithelium. An understanding of the anatomy and dynamic nature of this tissue is critical in understanding HPV entry. The epithelium has been classically described as having a multi-layer organization, with each layer having specific gene expression, protein forms, and cell architecture as the keratinocytes differentiate. The basement membrane serves several critical functions, including an influence on basal keratinocytes during differentiation and wound healing. Perhaps even more important is the barrier role of the

basement membrane that prevents keratinocyte growth into the dermis. Tumor cell invasion is marked by a proteolytic breakdown of the basement membrane via matrix metallo-proteinases produced by transformed keratinocytes (Chow, Broker, and Steinberg 2010).

The epithelium is in a state of constant turnover. HPVs are the only viral family whose infection requires actively proliferating cells (Hausen 2002; Doorbar et al. 2015). The basal keratinocytes are mostly quiescent, rarely entering the S phase for division. It is the parabasal keratinocytes, also known as transit amplifying (TA) cells, which actively divide. The TA cells typically divide every day or two for a couple months prior to entering the stages of terminal differentiation. As the cells move “up” through the various strata, various genes are up- or down-regulated, giving them specialized functions. When keratinocytes reach the most superficial stratum layer, the active cells are converted to an “oxidizing state” of cell death, and slough off (Chow, Broker, and Steinberg 2010).

HPV infection necessitates an injury or wound of the epithelium that allows for binding or uptake into basal cells (Chow, Broker, and Steinberg 2010; Doorbar et al. 2015; Doorbar 2006; Doorbar et al. 2012; Kines et al. 2009; Hausen 2002). The exact mechanism of initial binding is not completely understood, but heparin sulfate proteoglycans are thought to play a role. The role of alpha 6 integrin as a secondary receptor has also been documented. HPV particles are transported into the host cells via clathrin-coated endocytosis. The L2 capsid protein allows for transfer of the HPV DNA into the host nucleus following disassembly of the viral particles via endosomes (Doorbar 2006).

Initially, the viral genome exists as an episome without integration into the host genome. The E1 and E2 proteins play crucial roles in the replication of the viral genome. The E2 initiates the process by binding to the viral DNA, which in turn regulates the binding of E1 helicase to viral and cellular proteins necessary for replication. Replication of the viral genome occurs as the epithelial cells enter the S-phase. E2 also acts as a transcription factor and can regulate expression of viral oncogenes E6 and E7(Doorbar 2006).

Viral transcripts can be detected in cells in less than 12 hours post-infection, but it may take weeks before clinical papillomas are evident(Doorbar 2006). The most likely outcome of an HPV infection is prolonged latency. The latent infections are persistent and can be subclinical in nature. Active infection may follow latency as a result of immunosuppression or additional wounding. The low level of gene expression in latent infections make detection difficult and probably contribute to the lack of immune response by the host(Chow and Wang 2010; Doorbar 2006).

Once infection is established, proliferation of the host suprabasal cells is mediated by the expression of viral oncogenes E6 and E7. The E6 and E7 proteins push cells into the S-Phase. Cells continue to proliferate while E1 and E2 replicate the viral genome in these cells. The HPV infection prevents normal cell-cycle progression, which does not allow for terminal differentiation of the suprabasal cells as seen in health. Cell-cycle progression is stimulated by E7 associating with retinoblastoma protein (pRb). Cyclins A and E, which are necessary for viral DNA replication, are increased. Further, histone deacetylases, the AP1 transcription complex, and cyclin-dependent kinase inhibitors p21 and p27 are additional proteins involved in cell proliferation that E7 can associate with.



Studies have demonstrated that high levels of p21 and p27 can actually form inactive complexes with E7. This is interesting considering that expression of the viral oncogene E7 is important in high-risk HPV types that can lead to malignancies. In high-risk HPVs, the E7 and E6 proteins work synergistically to form a polycistronic mRNA species. In general, E6 has an anti-apoptotic effect on cells, allowing for continued proliferation even in cases of DNA damage and mutations. Individually, E6 can also regulate cell proliferation through its C-terminal PDZ ligand domain(Doorbar 2006).

Although lesions can form as a result of the increased cellular proliferation, infectious virions are only produced when the viral genomes are amplified and packaged. This process necessitates activation of the differentiation-dependent promotor region, which is typically located on the E7 ORF. Cellular signals, not viral genome amplification, are responsible for the activation. The result is an increase in early proteins necessary for replication, such as E1 and E2. While E4 and E5 do play roles in genome amplification, the specific details are not well known at this time.

Packaging of the infectious virions is the final stage of the viral productive cycle. Changes in mRNA splicing, along with termination of transcripts at late polyadenylation sites, signal for synthesis of the capsid proteins L1 and L2. Protein synthesis and RNA processing both help mediate the timing of capsid synthesis. In addition to L1 and L2, E2 is thought to play a role in the assembly of virions. The N- and C-termini of the L2 signal for localization to the nucleus of the cell where it binds to the promyelocytic leukemia (PML) bodies. Once the L2 has been localized to the nucleus, L1 assembles into capsomers. L2 and L1 associate with each other prior to virion release. Finally, E4

plays a role in disrupting the uppermost layers of the epithelium and allows for release(Doorbar 2006).

#### F. **DETECTION METHODS FOR HPV**

It is important to understand the various methods used to detect HPV in biopsies and surgical specimens. Cytological and histological examination is a basic way to detect HPV in samples of oral mucosa. Characteristics such as koilocytosis, perinuclear cytoplasm haloes, nuclear dysplasia, atypical metaplasia and binucleation may indicate HPV infection. Disadvantages of this technique include markedly reduced sensitivity compared to other methods and the inability to specifically determine the type of HPV present(Chaudhary et al. 2009).

There are numerous methods of HPV detection that rely on the presence of HPV DNA, mRNA transcripts, or translated viral proteins. Currently, none of the available tests are perfectly specific or sensitive. Technical difficulties and the need for fresh tissue are additional aspects of some available tests that make them less advantageous (Allen et al. 2010). Improved testing in the future can allow for a better understanding of the exact biologic mechanisms that differentiate HPV-positive OPSCCs.

Polymerase chain reaction (PCR) is one method that detects HPV activity base on the presence of HPV DNA. It is the most commonly used of all methods either on its own or in combination with other techniques. Success in detection of HPV in cervical cancers is well documented (Lerma Puertas et al. 2011). PCR has very high sensitivity with the ability to amplify a single sequence of DNA to detect as little as one copy of HPV DNA per cell(Chaudhary et al. 2009). DNA primers that are complimentary to

particular regions of the DNA allow for identification of various subtypes of HPV. It should be noted that mere presence of DNA, as detected by the PCR method, does not indicate the integration or expression of HPV genes. PCR also lack the ability to differentiate whether HPV DNA is specifically present in a sample's cancer cell versus the surrounding stromal tissue that is also part of the specimen unless laser capture microdissection (LCM) is employed. PCR can utilize either fresh frozen (FF) tissue or formalin-fixed paraffin-embedded (FFPE) samples(Allen et al. 2010).

HPV DNA that is used for PCR and other detection methods often comes from surgical specimens. Techniques that are less invasive and easy to perform would be advantageous as screening tools for oral HPV. Several groups have evaluated the predictability of HPV DNA extracted from exfoliated epithelial cells following an oral rinse(Smith et al. 2004; D'Souza et al. 2005; Chuang et al. 2008). One group specifically evaluated the ability of this technique to detect recurrence of disease in patients with previous HNSCCs. Results were mixed with 50% sensitivity and 100% specificity. The authors concluded that there could be promise in using this method for “surveillance and early detection of recurrence” (Chuang et al. 2008). Similarly, Smith et al. found that presence of oral exfoliated cells with high-risk HPV is a risk factor for HNSCC. Further, the authors concluded that an oral rinse test could potentially be predictive of an HPV-positive HNSCC (Bernard et al. 2010; Smith et al. 2004; Doorbar et al. 2015).

Hybrid capture II (HC II) is a nucleic acid assay that uses microplate chemiluminescent detection. dsDNA is denatured into single stranded DNA (ssDNA) which is then hybridized to specific HPV RNA probes. These DNA-RNA hybrids are reacted with antibodies on micro-well plate that emit light via cleavage of a

chemiluminescent substrate. The relative light units (RLU) are measured by a luminometer and calculations can be made to determine viral load(Doorbar et al. 2012; Chaudhary et al. 2009; Doorbar et al. 2015).

In situ hybridization (ISH) is another method that detects DNA in tissue samples. HPV DNA is localized utilizing complimentary nucleic acid probes, which can be directly viewed with microscopy and quantified colorimetrically. The probes can be radioactive, or contain dye- or florescent-labeled bases. Similar to PCR, the probes corresponding to specific subtypes of HPV may be used. The main advantage of ISH over PCR is the ability to directly visualize the physical status of HPV DNA. Specifically, ISH allows for the ability to differentiate between episomal and integrated viral DNA. PCR, however, is widely known to have superior sensitivity. Historically, the sensitivity of ISH was reported to be 10 viral copies per cell, but significant improvements have been made with the advent of new reagents and refined techniques(Doorbar et al. 2015; Allen et al. 2010; Chaudhary et al. 2009).

Blot hybridization is a third technique for DNA detection. Southern blot (SB) is the most widely used technique in this arena. Restriction enzymes are used to fragment the DNA and electrophoresis is completed. The DNA is then transferred to a membrane where specific probes hybridize the viral DNA. As with the PCR and ISH techniques, it is the design of the probes that can identify specific sequences of HPV DNA for identification. The SB technique also allows for differentiation between episomal and integrated DNA. Theoretically, the SB technique can demonstrates very high sensitivity, but studies have shown it to be significantly inferior to PCR overall(Bernard et al. 2010;

Allen et al. 2010; Chow, Broker, and Steinberg 2010; Doorbar et al. 2015; de Villiers et al. 2004).

The presence and integration of HPV DNA in itself does not indicate viral gene expression, which is necessary for malignant transformation. Reverse-transcriptase PCR (RT-PCR) can specifically identify E6 and E7 mRNA, which is indicative of biologically active HPV DNA. Reverse transcriptase (RT) creates a cDNA sequence, which can be amplified with the PCR methods already described. The combined utilization of mRNA with PCR allows for a very highly specific and sensitive test. The most commonly cited disadvantage to this technique is the need for FF tissue (Doorbar et al. 2015; Allen et al. 2010; Bernard et al. 2010; de Villiers et al. 2004). Smeets et al. recently successfully demonstrated use of RT-PCR with FFPE tissue samples when evaluating for E6 expression of HPV 16 (de Villiers et al. 2004; Smeets et al. 2007; CHOW, BROKER, and STEINBERG 2010; Doorbar et al. 2015). Despite being highly sensitive and specific, questions regarding the feasibility of utilizing RT-PCR in large studies still remain (CHOW, BROKER, and STEINBERG 2010; Allen et al. 2010). Recently, Kolokythas et al. reported a non-invasive detection of OSCCs by using brush cytology and RT-PCR. The highly sensitive and specific results suggest the potential for this non-invasive technique to be used as a clinical tool (Chow, Broker, and Steinberg 2010; Kolokythas et al. 2013; Doorbar et al. 2015).

The previously characterized ISH technique can also be utilized to identify mRNA by using RNA probes known as riboprobes. HPV 16 E6 and E7 mRNA have been identified with this technique when evaluating tonsillar tumor tissue samples (Chow, Broker, and Steinberg 2010; Wilczynski et al. 1998). The ability to detect mRNA, which

specifically denotes biologically active HPV, gives it a distinct advantage over the traditional method. However, when compared to RT-PCR, the sensitivity appears to be inferior (Doorbar et al. 2015; Allen et al. 2010).

Various tests already described here allow for detection of HPV mRNA, which specifically identifies biologically active viruses. Taking things another step further, there are techniques that take into account posttranscriptional regulation and oncogene expression. These direct assays identify the presence of oncoproteins involved with oncogenesis. Immunohistochemistry (IHC) is the most common technique of this type. First, an antibody is made for a specific protein. A second antibody linked to an enzyme allows for a calorimetric reaction that can be quantitatively analyzed microscopically with special software programs. While sensitivity has not adequately been quantified for these IHC techniques, superior specificity for HPV biologic activity is reported (Chow, Broker, and Steinberg 2010; Allen et al. 2010; Doorbar et al. 2015).

DNA microarray techniques utilize DNA probes labeled with radioisotopes or a fluorescent tag that hybridize specifically to complementary sequences in the sample DNA or RNA. Microarrays enable one to simultaneously analyze expression of thousands of genes and potentially identify differences between healthy tissue and tumor (Chow, Broker, and Steinberg 2010; Chaudhary et al. 2009; Jeon et al. 2004). Microarray techniques have been utilized to determine gene expression for various types of cancers including HNSCC (Doorbar et al. 2015; Golub 1999; Jeon et al. 2004). These techniques were used to create gene-expression profiles in breast cancer patients to improve prognostication (Hausen 2002; Van De Vijver et al. 2002). Martinez et al. compared gene expression of HPV-positive and HPV-negative OPSCCs with normal epithelium

using GeneChip, a microarray technique. Specific patterns of gene expression that may serve as potential biomarkers of OPSCCs were reported (Hausen 2002; Martinez et al. 2007).

#### G. **HPV AND ASSOCIATION WITH MICROBES**

The “oral microbiome” is the complex interaction of the oral biofilm, known as dental plaque, with other organisms and the various surfaces it colonizes. It is essentially made up of many different microbial ecosystems. The literature has identified numerous factors that affect oral biofilm formation such as inter-bacterial co-adhesion, pH, oxygen, and nutrients. To date, there are over 800 bacterial species identified that can colonize the oral cavity. Various surfaces, the presence or absence of disease, and cell-to-cell communications allow for the existence of many micro-niches. These micro-niches can have profound impact on the health of oral tissues and can even affect the properties of epithelial membranes (Doorbar et al. 2015; Filoche, Wong, and Sissons 2009).

The literature outlines atypical pattern of colonization, with particular bacteria labeled as “pioneers” and some as late-colonizers. Socransky et al. found that there are various complexes or clusters of bacteria that are typically seen together (Doorbar et al. 2015; Socransky et al. 1998). Properties of the biofilm can be altered through chemical signaling between species, exchange of genetic material, and quorum sensing. The resultant biofilm is complex and very dynamic, with members expressing phenotypes that vary from their typical planktonic behavior. Bacteria exist symbiotically or directly in competition with other species (Chaudhary et al. 2009; Hojo et al. 2009). Slots et al. has hypothesized that viruses may play a significant role in various types of periodontitis.

Viruses from the herpes family have been found closely associated with a number of periodontal pathogens. It is thought that viruses could affect the host immune response and inflict tissue damage, allowing for a change in the biofilm (Chow, Broker, and Steinberg 2010; Slots 2010; de Villiers et al. 2004; Doorbar et al. 2015; Doorbar et al. 2012).

Bacterial interactions and metabolism can affect properties of the mucosa. Ethanol metabolism into acetaldehyde (AA) by *Streptococci* species has been well documented. Metabolism of poly-cyclic aromatic hydrocarbons (PAHs) by various bacteria species such as *Haemophilus* and *Mycobacterium* has also been shown. It is the complex interactions of microbes, host immune response, anatomical niches, and modifying factors such as tobacco products that alter the biofilm lead to tissue destruction (Castro and Filho 2006; Schwartz 2012).

Mucosal damage, which is necessary for HPV entry, begins with the host immune response to the attached biofilm. The innate immune response to bacterial invasion results in production of pro-inflammatory cytokines that result in tissue damage. In addition to the host response, various bacterial species have the capability to directly cause tissue damage via collagenases and hyaluronidases. The resultant destruction of normally tight tissues junctions allows for deeper penetration of the bacterial species and HPV. *Streptococci* species and HPV can be internalized via L1 mediated heparan sulfate proteoglycan binding protein (HSGPBs). Once *Streptococcus* spp. are associated with oral keratinocytes there is adherence of histiocytes, Langerhan cells, macrophages, or B lymphocytes. Following internalization, the bacteria and HPV may lead to DNA damage, depending on the subtype of virus. This carcinogenic effect may be enhanced with



alcohol and tobacco products. Anatomically, the oropharynx has associated lymphoid aggregates closely associated with the epithelium which could provide an exaggerated response to biofilm formation(PhD 2014; Schwartz 2012).

## **II. MATERIALS AND METHODS**

### **A. PARTICIPANTS**

This was a pilot study of 14 human subjects that could serve as a foundation for a larger cohort study in the future. Participants were recruited from the University of Illinois College of Dentistry. All subjects were 18 years or older and had a comprehensive periodontal examination. The comprehensive examination including a head and neck cancer screening, intraoral examination of hard and soft tissues, periodontal charting (6 sites per tooth), and a full-mouth series of radiographs. Periodontal diagnosis was based on the Armitage Classification(Castro and Filho 2006; Armitage 1999). Subjects who had a diagnosis of generalized moderate to severe chronic periodontitis were included in the study. Subjects with aggressive or necrotizing periodontitis, periodontal abscesses, or endodontic-periodontic lesions were excluded. A complete medical history was taken. Participants were only included if they were classified as Class I or II according to the American Society of Anesthesiologists Physical Status Classification. Subjects were excluded if they had uncontrolled systemic disease. All participants completed a survey regarding oral health and social history. Subjects with current or former use of tobacco and alcohol were included. Participants who had taken antibiotics or had periodontal treatment within the 3 months prior to the study were excluded. All participants reviewed and signed a written consent form. The study protocol reviewed approval by the IRB (2012-1030).

**B. SAMPLE COLLECTION**

Collection of bacterial plaque was taken from sulcus of periodontally diseased teeth (attachment loss  $\geq 3$  mm exhibiting clinical signs of inflammation) and periodontally healthy teeth. A paper point was placed in the sulcus for 30 seconds. The paper point was removed and placed directly into buffered solution. Bacterial samples were also taken from various sites in the oral cavity including the buccal mucosa, lateral border of the tongue, oropharynx, and the attached gingiva adjacent both periodontally diseased and healthy teeth. Samples were obtained by swabbing the sites with cotton tipped applicators. The cotton swabs immediately placed into buffered solution. Keratinocyte samples were taken from the buccal mucosa, lateral border of the tongue, oropharynx, and attached gingiva at periodontally diseased and healthy sites. Cytology brushes were vigorously manipulated over each area and then placed into medium.

**C. DNA PURIFICATION**

DNA was purified with the Gram-positive DNA purification kit MGP04100 from Epicentre.

**D. QUANTITATION OF 16 sRNA LEVELS**

Genomic DNA was amplified using the Earth Microbiome Project barcoded primer set, adapted for the Illumina HiSeq2000 and MiSeq. The 5' region of the 16S rRNA gene (27F-534R) was amplified and primers with primers that included the Illumina flowcell adapter sequences. The reverse amplification primer also contained a twelve base barcode sequence. (Castro and Filho 2006; Caporaso et al. 2012; Caporaso et al. 2010). Each 25ul PCR reaction contains 12ul of MoBio PCR Water (Certified

DNA-Free), 10ul of 5 Prime HotMasterMix (1x), 1ul of Forward Primer (5uM concentration, 200pM final), 1ul Golay Barcode Tagged Reverse Primer (5uM concentration, 200pM final), and 1ul of template DNA. The conditions for PCR are also follows: 94°C for 3 minutes to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. The PCR amplifications are done in triplicate. The amplifications are then pooled. Amplicons were quantified using PicoGreen (Invitrogen) with a plate reader. After combining the amplicons into a single tube, UltraClean® PCR Clean-Up Kit (MoBio) is used, and then quantified by Qubit (Invitrogen). Following quantification, the molarity is determined with a 30% PhiX spike. Amplicons are then sequenced in a 251bp x 12bp x 251bp MiSeq run using custom sequencing primers and procedures described by Caporaso and colleagues (Castro and Filho 2006; Caporaso et al. 2012; Prabhu and Wilson 2013; PhD 2014).

#### E. CONVERSION OF 16sRNA TO LEVELS OF TAXONOMIC UNITS

Sequence data were processed using the software package QIIME (PhD 2014; Caporaso et al. 2010; Castro and Filho 2006; Prabhu and Wilson 2013). Raw sequence data from the Illumina MiSeq instrument were initially de-multiplexed using a separate index read using the split-libraries function, with default quality trimmed (split\_libraries\_fastq.py). Sequences from the entire dataset were then clustered into operational taxonomic units (OTU) - groups of sequences of at least 97% similarity - using the pick OTU function, implementing the UCLUST clustering algorithm (pick\_otus.py). From this clustering, a biological observation matrix (BIOM) - a table of samples by taxa (OTU) consisting of number of sequences from each sample of each

taxon - was generated. The BIOM was further processed to a series of BIOMs at various taxonomic levels (phylum, class, order, family, genus) using the split\_otu table script within QIIME (split\_otu\_table\_by\_taxonomy.py).

#### F. **STATISTICAL ANALYSIS OF MICROBIAL POPULATION**

For determination of differential representation of microbial species or genera in different sample sets we used STAMP(Prabhu and Wilson 2013; Parks et al. 2014; Castro and Filho 2006; PhD 2014). STAMP is a graphical software package that allows one to compare the numbers of bacteria or any species in one group versus one or more other groups. It analyzes the data to provide a list of species that are present at different number in the comparison and provides a statistical analysis of the probability of the difference being real. It is optimized for analysis of microbial datasets where many if not most entries are frequently zero. It also allows correction for multiple testing.

#### G. **TRYPAN BLUE DYE EXCLUSION**

Cell viability is yested using a 1:20 dilution of trypan blue (0.25%) (Sigma-Aldrich Chemical, St Louis, MO). A Leica inverted microscope along with a calibrated micrometer was used for cell counting (W. Nuhsbaum Inc., McHenry, IL).

#### H. **MICONUCLEUS ASSAY**

Micronucleus presence was assessed with a vital nuclear stain *Hoechst 33342* (1 µg/mL) (Sigma Chemical, St Louis, MO). Fixation completed using 1% para-formaldehyde for 2 - 3 at 4°C. Cells are rinsed with phosphate buffered saline (1X) (PBS), Mayer's hematoxylin (Certified hematoxylin (1.0 g/l), sodium iodate (0.2 g/l),

aluminum ammonium sulfate·12 H<sub>2</sub>O (50 g/l), chloral hydrate (50 g/l) and citric acid (1 g/l) (Sigma-Aldrich Chemical, St. Louis, MO).. Micronuclei are categorized by the size of micronuclei (1/16th and 1/3rd of the mean diameter of the main nuclei); being non-retractile; not linked or connected to the main; the nuclei touches but does not overlap the main nuclei; stains with same intensity as main nuclei. (Allen et al. 2010; Fenech 2000).

#### I. **PRODUCTION OF HUMAN PAPILLOMA VIRUS PSEUDOVIRUS (PsV)**

The 293TT cells are essentially an adenovirus transformed cell line. PsV production and an Optiprep purification method using overnight incubation of crude cell lysate at 37°C (Mehanna et al. 2012; Buck and Thompson 2007). A map of HPV 16 PsV packaging plasmid (p16sheLL for HPV and pCDNA-GFP for GFP gene) as described by various authors can be found in the literature (Chaturvedi et al. 2011; Buck et al. 2006). Visualization is possible due to a green fluorescent protein (GFP).

#### J. **PSEUDOVIRUS INFECTION**

Pseudoviral like particles (PsV) were added to collagen coated plates containing 293TT or hTERT HOK cells at a 50% - 60% confluence ( $5 \times 10^5$  cells). The PsV (50 µL of a 1:1000 crude cell lysates dilutions) is added and incubated for 24 hours. Entry was monitored a minimum of 7 days 7 days. Prior to PsV addition a titration assay (200-100-50-25-10-5 microliters) was conducted for each cell line to determine maximum GFP expression. (Chaturvedi et al. 2011; Buck et al. 2004; Buck et al. 2005).

## K. QUANTITATION OF PSEUDOVIRUS ENTRY

Two methods were used to assess HPV 16 entry (PsV). The number of fluorescent epithelial cells was counted in dark field utilizing a minimum of three photomicrographs (40×). A bright field photomicrograph was used to count the total number of cells. Fluorescent unit values were calculated by dividing experimental by the control untreated background value raw numbers that are obtained from the fluorometer (Mehanna et al. 2012; Buck et al. 2004; Allen et al. 2010; Buck et al. 2005; Chaturvedi et al. 2011)

### III. RESULTS

#### A. HPV-16 ENTRY ASSAYS

Figure 1 compares HPV-16 entry into human buccal mucosa keratinocytes in smoker and non-smokers subjects without periodontitis. The non-smokers had a mean entry of 6.4% (+/- 2.6%). Subjects who smoked had a greater mean entry of 72.0% (+/- 3.8%).

**Figure 1 – HPV-16 Entry Buccal Mucosa**

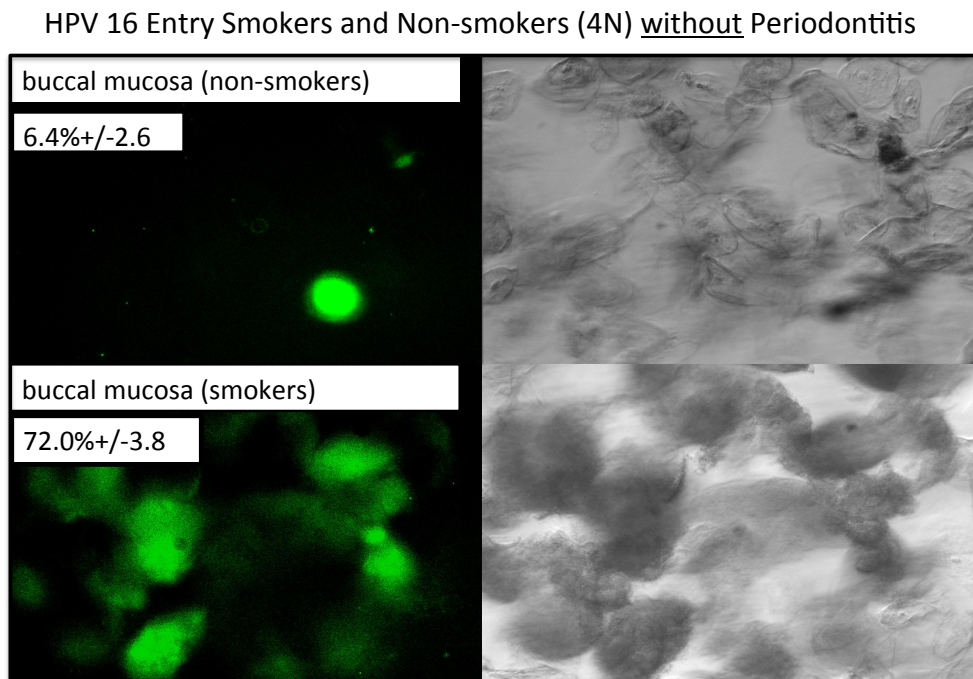




Figure 2 compares HPV-16 entry into human oropharynx keratinocytes in smoker and non-smokers subjects without periodontitis. The non-smokers had a mean entry of 23.5% (+/- 8.0%). Smokers had a mean entry of 65.5% (+/- 7.2%) which was more than two times that seen in the non-smokers.

**Figure 2 – HPV-16 Entry Oropharynx**

HPV 16 Entry Smokers and Non-smokers (4N) without Periodontitis

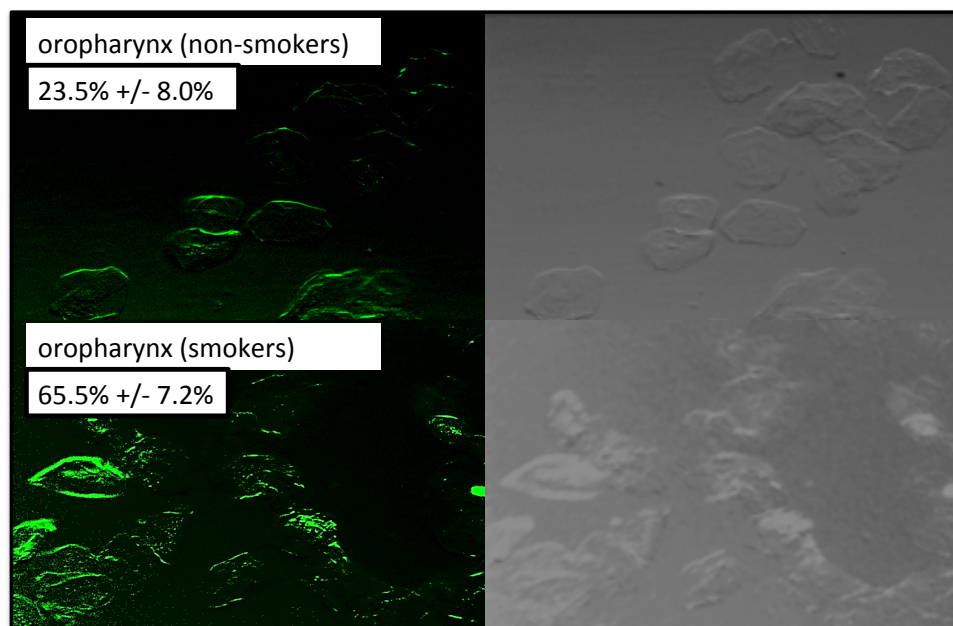


Figure 3 demonstrates HPV entry at various sites in the oral cavity for smokers and non-smokers. Generally, there was greater HPV-16 entry in smokers versus non-smokers, as demonstrated at the oropharynx and buccal mucosa sites. The keratinized gingiva demonstrated greater entry in the non-smoker subjects than the smokers. The buccal mucosa site for smokers had the highest overall entry of HPV-16.

**Figure 3 – HPV-16 Entry at Various Oral Sites**

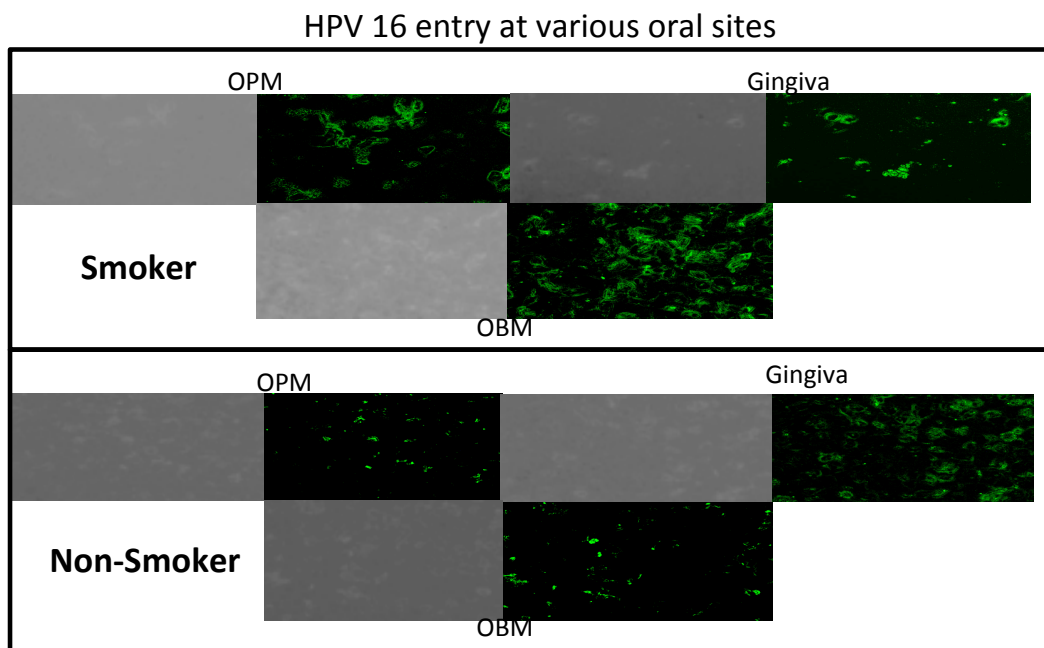
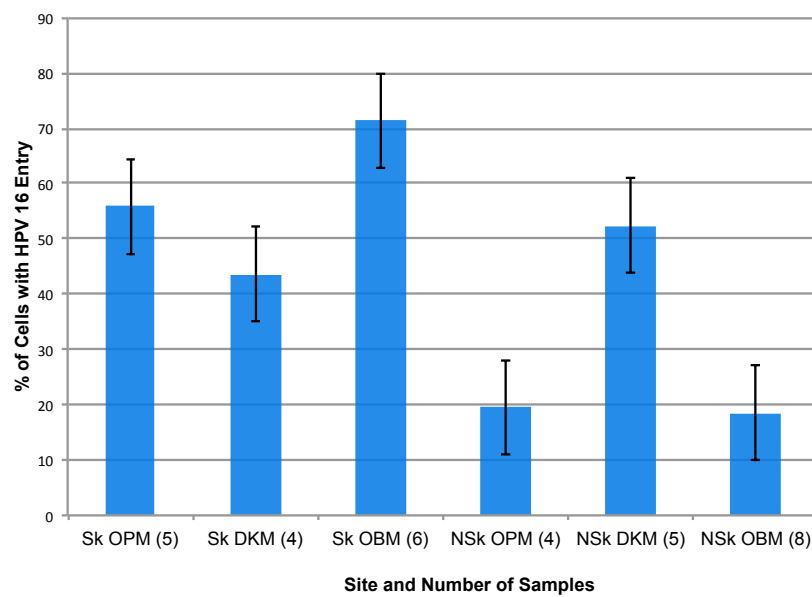


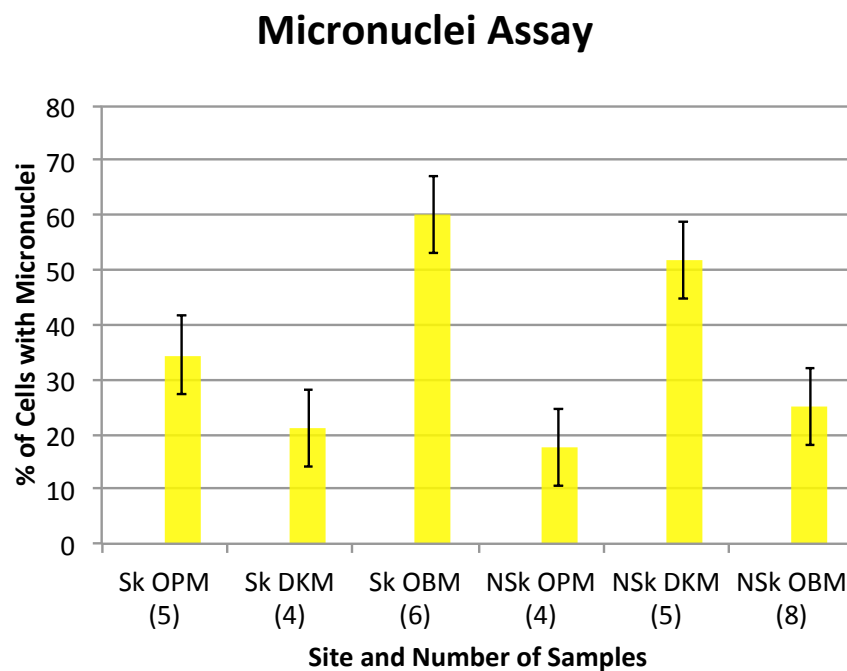
Figure 4

**HPV 16 Entry Assessed Through In vitro Assay**

## B. MICRONUCLEI ASSAY

The results from the micronuclei assay are shown in Figure 5. The keratinocytes from smokers generally had a higher percentage of micronuclei present compared to non-smokers, with the exception of the keratinized gingiva site compared to smoker which was also significantly increased. Cells from the buccal mucosa in smokers had approximately 60% of cells with micronuclei present, which was the highest percentage of any group.

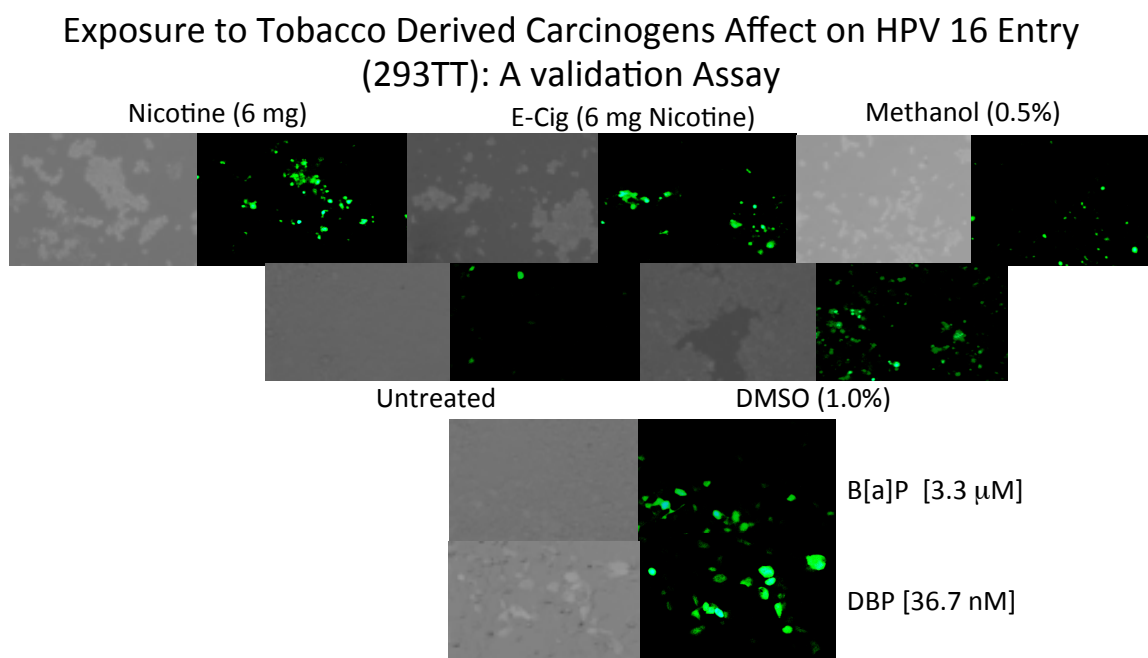
**Figure 5**



### C. HPV-16 ENTRY IN PRESENCE OF TOBACCO PRODUCTS

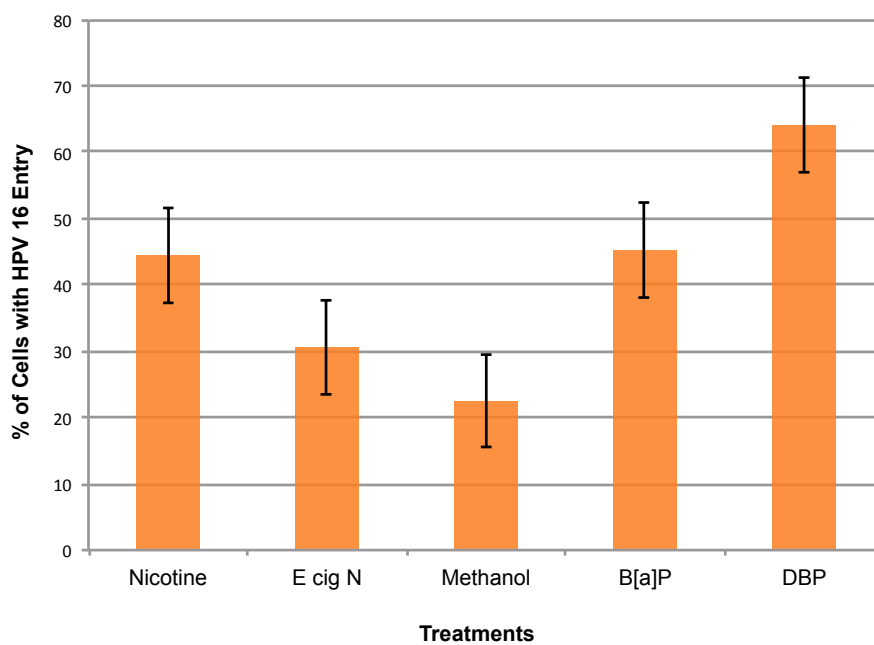
Figures 6 and 7 show the results from a validation assay looking at HPV-16 entry when human keratinocytes cells are exposed to tobacco-derived carcinogens *in vitro*. All of the tobacco-derived products demonstrated increased HPV-16 entry compared to the untreated cells.

**Figure 6 – HPV-16 Entry In Presence of Tobacco Products**



**Figure 7 – Derived Carcinogens Affect on HPV-16 Entry**

### Derived Carcinogens Affect on HPV 16 Entry (293TT)



#### D. 16S rRNA MICROBIAL IDENTIFICATION

Figures 8, 9, and 10 demonstrate bacterial species with increased presence in subjects with periodontitis versus subjects without periodontitis. Participants were non-smokers. In the oropharynx, there was a significant increase in *Novoshingobium spp.* for periodontitis subjects compared to subjects without periodontitis where this species was not found (Figure 8). A similar trend was found for both *Prevotella spp.* (Figure 9) and *Rothia spp.* (Figure 10). While *Prevotella spp.* was found in subjects without periodontitis, it was found more commonly and in greater amounts in the periodontitis subjects (Figure 9). *Rothia spp.* was also present in both groups but was significantly greater in the periodontitis subjects (Figure 10).

Figure 8

# *Oropharynx - Novosphingobium spp.*

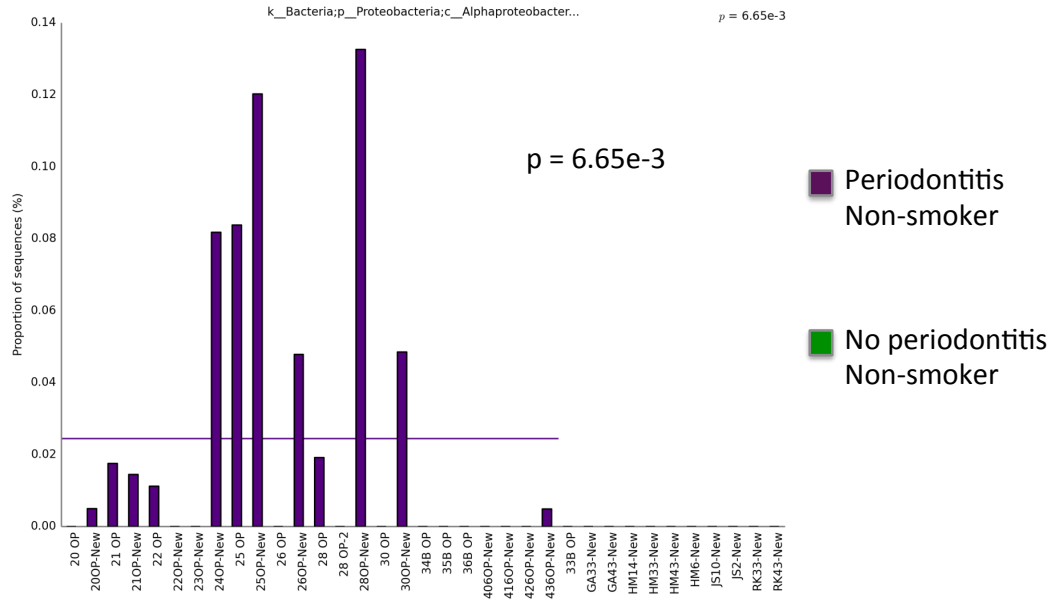




Figure 9

# Oropharynx – *Prevotella* spp.

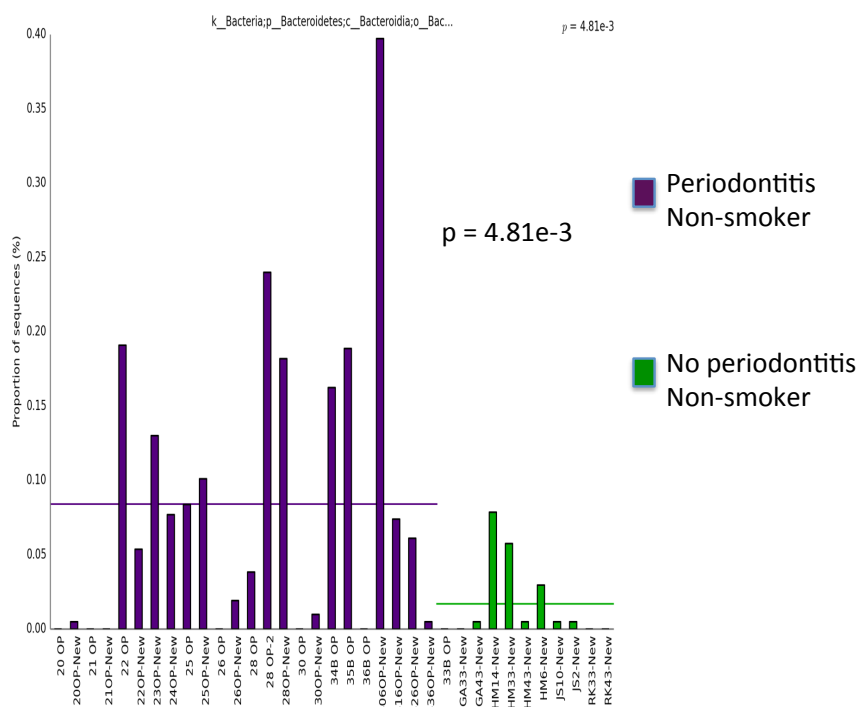
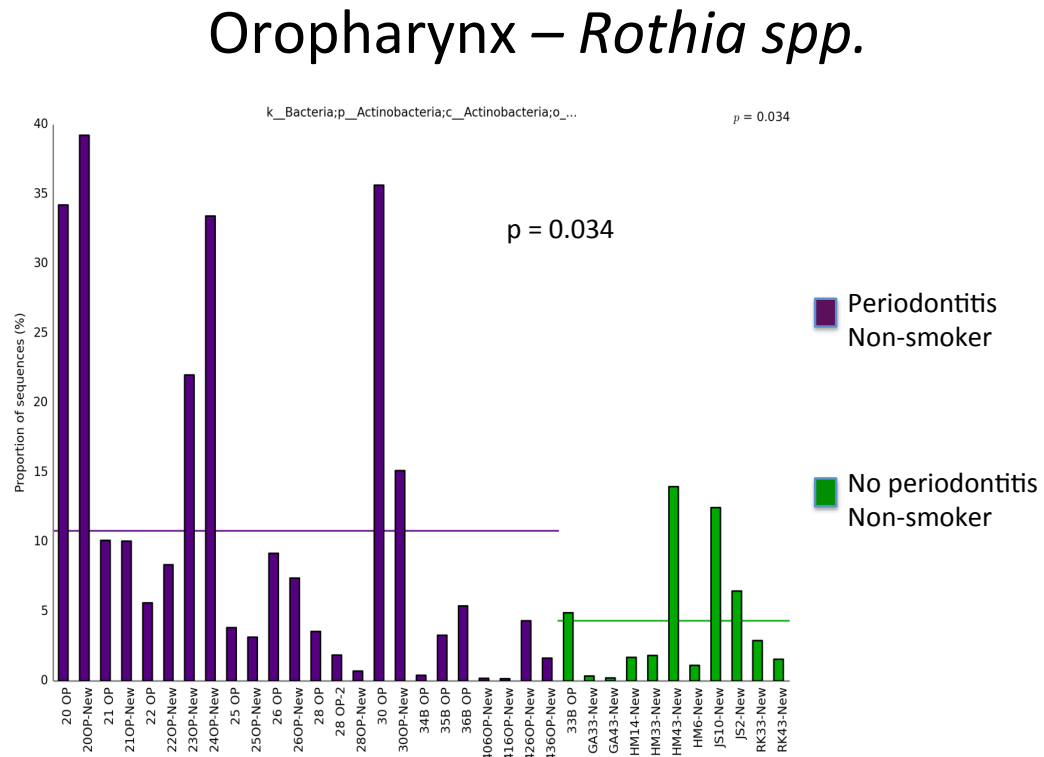


Figure 10



There were numerous bacterial species that were markedly reduced or not found in the oropharynx of subjects who were smokers compared to non-smokers (Figures 11 – 14). *Nesisseria spp.* was found in subjects who were non-smokers but was not present in the oropharynx of smokers (Figure 11). Similarly, very reduced amounts of *Haemophilus spp.* were found in a several subjects who were smokers, but it was present in all non-smoking subjects (Figure 12). There were a small number of smoking subjects that had elevated levels of *Porphyromonas spp.* and *Gamellaceae spp.*, however, all non-smokers had the presence of these microbes, mostly at much higher levels (Figures 13 and 14).

**Figure 11**

## Oropharynx - *Neisseria spp.*

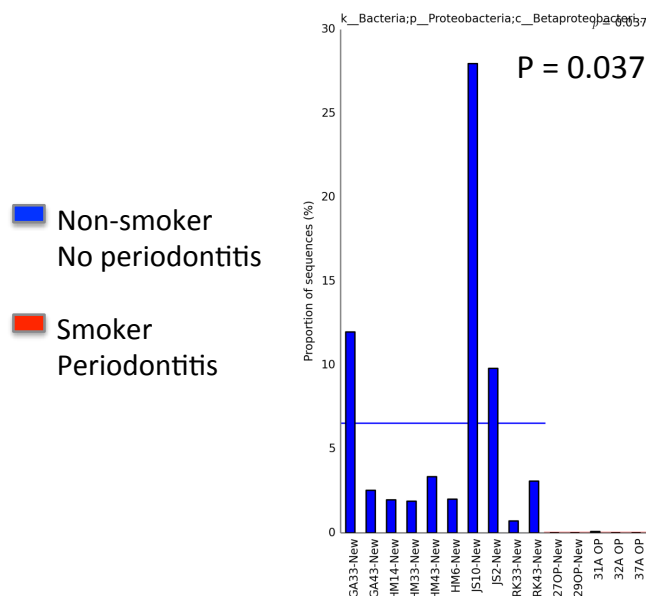


Figure 12

## Oropharynx – *Haemophilus* spp.

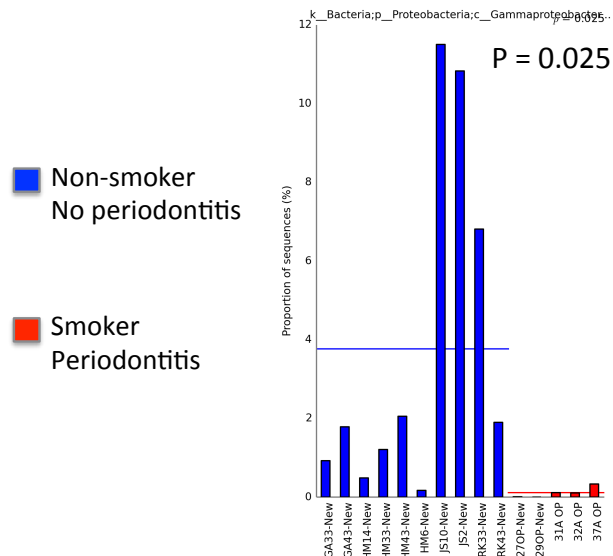


Figure 13

## Oropharynx - *Gemellaceae* spp.

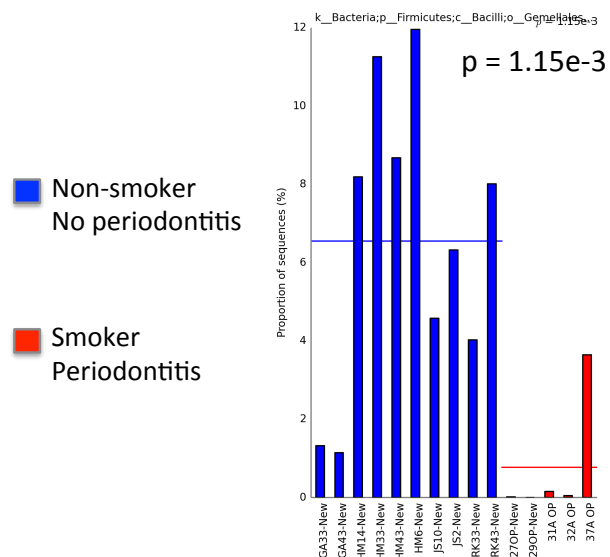
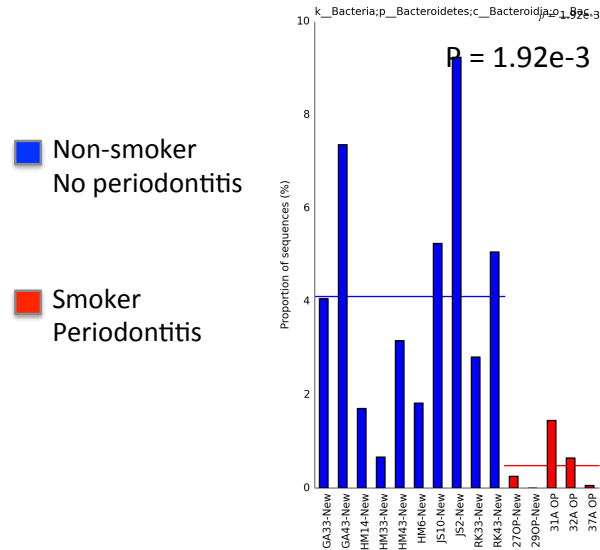


Figure 14

# Oropharynx - *Porphyromonas* spp.



#### **IV. DISCUSSION**

Results from the HPV-16 entry assays show significantly increased entry into oral keratinocytes taken from the buccal mucosa and oropharynx in smokers compared to non-smokers. These results coincide with the known clinical risk profile for HPV-16 infection. Chronic exposure to tobacco smoke and alcohol are well established as risk factors for HNSCCs. Until recently, the literature has not been conclusive in regards to the contribution of smoking on HPV associated OPSCCs. In 2008 Gillison et al. reported increased risk of HPV positive cancers associated with increased sex partners and use of marijuana. There was no association of increased risk with tobacco or alcohol use, except for the HPV negative HNSCCs (Doorbar et al. 2015; Gillison et al. 2008; Allen et al. 2010; Chaturvedi et al. 2011). More recently, a study analyzing the 2009-2010 NHANES data shows a significantly increased prevalence of oral HPV infection in current smokers compared to former or non-smokers, especially in women. The significance of smoking was also dose-dependent, with a current smoking habit of >20 cigarettes per day carrying a higher prevalence than those smoking less (Hennessey, Westra, and Califano 2009; Gillison et al. 2012). We hypothesize that the tobacco products act to alter the microbiome and which leads to changes in the nature of the epithelial barrier. These changes in the epithelium may lead to increased entry and eventual establishment of chronic HPV infections.

The keratinized gingiva demonstrated the highest entry of HPV-16 in our study. This is not supported by the literature regarding clinical site specificity that is seen with HPV-positive HNSCCs. It is important to consider that our study simply evaluates entry into the cells. It does not evaluate integration of the HPV genome into the host cell, nor

does it investigate malignant transformation. The entry of the virus is one piece of the overall “big picture”. Although increased entry was seen at the keratinized gingiva, a very low-risk site for the clinical presentation of HPV-positive OPSCCs, we do not feel this is related to the clinical presentation of disease. We observed an increase among non-smokers but in a previously diagnosed inflammatory state associated with periodontitis. This result suggests that this type of inflammatory condition is associated with tissue degradation which is part of a DNA damage profile that requires further study. It is also possible that results of the HPV-16 entry assays would be different if there were larger numbers of participants.

In this study we found that there was an overall increase in microbial diversity in patients with periodontitis when compared to patients without periodontitis. Specifically, there was a significantly increased presence of *Novoshingobium spp.*, *Prevotella spp.*, and *Rothia spp.* in periodontitis patients. A recent study of subgingival plaque utilizing 16S pyrosequencing found that subjects with periodontitis had more bacterial diversity compared to patients without periodontitis, supporting our current findings (Allen et al. 2010; Camelo-Castillo et al. 2015).

There were distinct differences in the microbiome found between smokers and non-smokers. We found that subjects who were smokers with periodontitis had significantly lower levels or lacked presence of *Nesisseria spp.*, *Haemophilus spp.*, *Porphyromonas spp.* and *Gamellaceae spp.*. Overall, these findings demonstrated a general reduction in the bacterial diversity presents for the smokers. Castillo et al. found similar results in a study investigating the microbiota in periodontitis patients. The study showed that there was reduced microbial diversity in smokers who had periodontitis compared to patients

who were not smokers. Specifically, there was a significantly higher abundance of *Porphyromonas* in the non-smoking group. These findings correlate with what we found in this current study (Camelo-Castillo et al. 2015).

We believe that this study is the first to evaluate the effects of smoking and the oral microbiome on entry of HPV-16 into human oral keratinocytes. One of the greatest strengths of this study relates to the fact that it is a human study. The diagnosis of periodontitis was also based on a comprehensive exam, which included full-mouth periodontal measurements and a full mouth series of radiographs. Microbial analysis was completed using the 16S rRNA pyrosequencing technique, which allows for a very in-depth analysis of all bacteria present in the microbiome sample. The main limitation to this study was the small number of participants and confounding variables. A larger patient population would allow for more samplers to be taken. An increased number of subjects would also allow for better comparison between different age groups, smoking statuses, and periodontal conditions. Future studies will need to specifically evaluate the biology of bacteria identified and their potential roles in altering membrane properties of the epithelial cells. The microbial-epithelial interactions may lead to more insight on the exact mechanism that the microbiome may influence HPV-16 entry into the keratinocytes.



## V. CONCLUSION

This study investigated the effects of smoking and the microbiome on entry of HPV-16 into human oral keratinocytes. Subjects who were smokers had a mean entry of 65.5% (+/- 7.2%) at the oropharynx site, which was more than two times that seen in the non-smokers (23.5 +/- 8.0%). Subjects who were smokers and had a diagnosis of moderate to severe periodontitis had significantly less diverse microbial populations with reduced presence of *Nesisseria spp.*, *Haemophilus spp.*, *Porphyromonas spp.* and *Gamellaceae spp.*. Subjects with periodontitis who were not smokers demonstrated more diversity of the microbiome compared to non-periodontitis subjects with increased presence of *Novoshingobium spp.*, *Prevotella spp.*, and *Rothia spp.*. It is plausible that HPV-16 entry is affected by exposure to tobacco smoke and changes to microbial ecology that are site specific.

Tobacco smoke association with enhanced periodontal disease risk also is associated with DNA damage and a generalized change in oral microbiome diversity, number and bacterial genera.

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