Influence of Smoking on Microbial Diversity

BY

HARRISON EDWARD MACKLER
B.S., Clark University, Worcester, Massachusetts, 2007
D.M.D., Harvard University, Boston, Massachusetts, 2012

THESIS
Submitted as partial fulfillment of the requirements for the degree of Master of Science in Oral Sciences in the Graduate College of the University of Illinois at Chicago, 2015

Chicago, Illinois

Defense Committee:
Joel Schwartz, Oral Medicine & Diagnostic Sciences, Chair and Advisor
Guy Adami, Oral Medicine & Diagnostic Sciences
Lin Tao, Oral Biology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>A. Human Oral Microbiota</td>
<td>1</td>
</tr>
<tr>
<td>i. Healthy Oral Microbiome</td>
<td>2</td>
</tr>
<tr>
<td>ii. Techniques for Identification</td>
<td>3</td>
</tr>
<tr>
<td>iii. Site Specificity</td>
<td>4</td>
</tr>
<tr>
<td>iv. Age and Tissue Site and Oral Microflora Diversity</td>
<td>9</td>
</tr>
<tr>
<td>B. Smoking</td>
<td>15</td>
</tr>
<tr>
<td>i. Periodontal Disease, Tobacco Smoke, and Biofilm Modification</td>
<td>18</td>
</tr>
<tr>
<td>C. Oral Cancer</td>
<td>22</td>
</tr>
<tr>
<td>i. Oral Microbiome and Cancer</td>
<td>24</td>
</tr>
<tr>
<td>2. Methods</td>
<td>28</td>
</tr>
<tr>
<td>A. Patient selection</td>
<td>28</td>
</tr>
<tr>
<td>B. Periodontal exam and diagnosis</td>
<td>28</td>
</tr>
<tr>
<td>C. Sample collection</td>
<td>28</td>
</tr>
<tr>
<td>D. Quantitation of 16 sRNA levels</td>
<td>28</td>
</tr>
<tr>
<td>E. Conversion of 16sRNA quantitation to levels of taxonomic units</td>
<td>29</td>
</tr>
<tr>
<td>F. Statistical Analysis of Microbial population numbers</td>
<td>29</td>
</tr>
<tr>
<td>3. Results</td>
<td>30</td>
</tr>
<tr>
<td>4. Discussion</td>
<td>33</td>
</tr>
<tr>
<td>5. Works Cited</td>
<td>36</td>
</tr>
<tr>
<td>6. Vita</td>
<td>47</td>
</tr>
</tbody>
</table>
## FIGURE LIST

<table>
<thead>
<tr>
<th>Figure Number</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Microbiome changes in smokers vs. non-smokers (tongue)</td>
<td>40</td>
</tr>
<tr>
<td>2.</td>
<td>Microbiome changes in smokers vs. non-smokers (oropharynx)</td>
<td>41</td>
</tr>
<tr>
<td>3.</td>
<td>Microbiome changes in periodontitis vs. non-periodontitis</td>
<td>42</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OSSC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>Spp</td>
<td>Species</td>
</tr>
</tbody>
</table>
SUMMARY

Head and neck cancers will kill 8,000 people every year in the United States, in addition to affecting 41,000 new cases. While treatment for oral cancer typically include a combination of surgery, radiation, and chemotherapy, these methods may cause many deleterious effects to the patient and can encourage some to forgo treatment until it is too late and the cancer is in an incurable, advanced stage. As pro-carcinogens continue to become more common, as in tobacco smoke, environmental factors and food, risk factors for head and neck and other cancers rise. There is a clear need to identify patients at highest risk for cancer in order to reduce their threat and begin a preventative treatment as quickly as possible. Surrogate markers for toxic oral bacteria, such as tooth loss, and their link to different cancers suggest that oral bacteria have effects on oral and other cancer rates and additional diseases.

Objective: We propose to examine bacteria at different sites in the oral cavity to determine if there are bacteria species and genera associated with tobacco exposure and periodontitis.

Methods: We have sampled bacteria on healthy and diseased gingival surfaces, healthy and diseased sulvi and the oropharynx of n=24 subjects.

Results: The diversity of the oral microbiome in the tongue and oropharynx were found to increase in subjects with periodontal disease compared to healthy controls. Conversely, the diversity decreased in these subjects with periodontal disease in smokers versus non-smokers. Qualitatively, dysbiosis is suggested to increase in the face of both periodontal disease and smoking. These results provide novel mechanisms into the genesis of oral cancer in patients with periodontal disease and/or smokers.
1. Introduction

A. Human Oral Microbiota

There is a growing acknowledgement that the oral microbiome is an important complex of human associated microbial habitats that influence oral health through a process of homeostasis and/or dysbiosis (Hajishengallis 2014; Jiao et al. 2014).

It is also clear that the oral cavity is a window to changes in environment. The oral cavity constituents such as the microbiome, host cells, and host tissues are under constant influence from dietary constituents and habits that produce a profound change in cell viability. This occurs as a function of select chemical compositions and air quality chemistry that is interactive with a variety of human cell types. An important mediator in this system is the oral microbiome that possesses a physiology, chemistry, and biochemistry that is in adaptive response and seeks to function in concert with adjacent humans cells and tissues to achieve a homeostatic; symbiotic or dysbiotic state. A homeostatic situation exists between microbes and host tissue after an equal balance of requirements for survival is achieved without loss of a sustainable viable; energy functional state and form for microbiome or host tissue. A symbiosis is achieved when microbiome or host tissue achieves a state of advantage over the other, although without debilitation of either host tissues or microbiome or losses in viability of either. Dysbiosis is an unbalanced state that either microbiome or host tissue to survive produces a disadvantage to host tissues or microbiome. This process results in loss of a viable functional state and deterioration of oral mucosa or microbiota to produce an increased risk for disease.
In other words, a loss of a “healthy” homeostatic state is expected to be significant because it characterizes the ecology and environment for the presence of disease (e.g., signs and symptoms). We also anticipate improvements in technology will also demonstrate mechanism or pathogenesis in terms of a metabolic, physiologic, inflammatory and/or inappropriate genetic expression leading to a clinical relevant disease.

The underlying concept to our study is to examine for presence of these interactive states between microbiome and host tissue and assess quality of interaction by quantifying diversity of microbiome at a variety of host tissues in the presence of environmental influence from tobacco smoke chemicals.

There are two different forms of exposures from the environment we consider in our study: Environment exposures in different living conditions or release of a myriad of chemicals and particles that function as DNA damaging agents that affect both microbiome and host oral tissues because of a tobacco smoking habit.

i. **Healthy Oral Microbiome**

To evaluate chemical influences the microbiome, which consists of at least 700-800 different taxa, we record diversity changes (evenness, richness/abundance) at tissue sites (He et al. 2015). In addition, an observation of changes in diversity is expected to enhance our understanding of physical and chemical relationships between microbes and host tissue. We expect our approach will improve understanding and lead to eventual control of inappropriate debilitation and tissue breakdown between microbiome and oral mucosa tissues. In parallel with this analysis we further anticipate a better understanding of the microbiome chemical metabolic activity to be associated with diversity change at different oral tissue sites.
It is also well documented that a variety of microbes are associated with infectious
diseases. These include dental caries; pulpitis; alveolar osteitis; tonsillitis, and gingival
diseases (gingivitis and periodontitis) (Dewhirst et al. 2010). It is also reported that dental
caries and periodontal sites present with a divergent change as to level of diversity as
infection becomes prevalent at these tissues. That is, caries microbial diversity decreased
while periodontal disease microbial diversity increased. This increase in diversity associated
with subgingival microbial diversity becomes important when we consider the effects of
tobacco on periodontal tissue association with a microbial biofilm (Griffen et al. 2012; Gross

Among the 700-800 different taxa of oral bacteria there are 11 phyla and at least 25-77
genera that are generally associated with a normal oral microbiota (oral microbiome). For
example, phyla include *Firmicutes* (52.3%); *Proteobacteria* (19.7%); *Bacteriodetes* (15.6%);
*Actinobacteria* (7.3%); *Fuseobacteria* (5.0%); with less than 1.0% for *SR1, TM7, Cyanobacteria, Spirochaetes, Teneicutes, and Synergistetes* (Ahn et al. 2012). In another
study using a similar 16S rRNA gene sequence and the Human Oral Microbiome Database
(HOMD) (www.homd.org) produced similar results (Chen et al. 2010). Although, Chen et.al.,
identified 619 taxa in 13 phyla and in addition, to phyla identified above *Chyamydiae* and
*Chloroflexi, and Euryarchaeota* were also noted to be present in “healthy” oral microbiome.

**ii. Techniques for Identification**

A reason for differences in relative abundance recorded for different phyla is found in
this study by Dewhirst et al. (2010). This group showed 1,179 taxa; of these 24% were
identified and only 8% were cultivated while 68% are not cultivable for further identification
(Dewhirst et al. 2010). Percentage identification differences of phyla and genera are a derivative of the technology used for evaluation. Studies from the 1980 through early 2000’s largely focused upon cultivation of microbes under laboratory settings and this approach is restrictive as to identification of novel microbes (Socransky et al. 1998; Parahitiyawa et al. 2010). A later innovation was a DNA-DNA hybridization checker board approach (Mager et al. 2003; Paster et al. 2006), and most recently a 16S pyrosequencing has been used (Griffen et al. 2012; Chen et al. 2014; Camelo-Castillo et al. 2015). Each of these approaches has advantages and disadvantages. For example the early approach while highly specific only identified microbes capable of growing colonies under laboratory conditions and this eliminated many facultative aerobes with special growing conditions or anerobes which can be oxygen sensitive. The DNA-DNA hydrid checkboard approach added to our knowledge of species and enhanced our understanding of microbial abundance and change at different sites but the level of consistency and assessment for diversity because many are low richness species cannot be detected at a particular site.

In this current state of knowledge we are required to determine presence of a common core for a “healthy” microbiome and to evaluate change from the environment with a 16S pyrosequencing to provide the greatest level of consistent specificity at least for phyla and genera levels and it is for this reason we use this approach in this study (Griffen et al. 2012).

iii. Site Specificity

It is assumed the oral cavity consists of different microbial ecology sites; such as, dentition, gingiva attached mucosa, gingiva sulcus, lateral border of the tongue, base of the tongue, buccal mucosa, vermillion border of lip, hard palate and soft palate. (Mager et al.
2003; Aas et al. 2005). Our samples were taken from several of these sites. However, it is unclear whether repeated sampling from identical sites will provide the identical distribution of microbe distribution of phyla. It is expected that microbiome in select microenvironments will differ because of exposure to saliva flow, nitrosation process, and anatomical configuration of host tissues. For example microbe biofilm accumulation will occur at sites with low salivary flow, high nutrient accumulations, in crevices bordered by papillae or lymphoid tissues (Aas et al. 2005).

In another study 141 species were identified and 60% were cultivated. In contrast to the above studies *Gemella, Granulicatella, Streptococcus*, and *Veillonella* were identified using 16S rRNA sequences but the authors also stated that different sites had 20 to 30 different predominant species but in the oral cavity there is a predominant microbial flora that is highly diverse for site and subject and this creates a high degree of inter-individual variation (Aas et al. 2005).

In a recent study that used 16S pyrosequencing, another set of predominant microbes phyla and genera were identified. Zaura et al. (2009) obtained samples from tooth surfaces, cheek, hard palate, tongue and saliva and found 500 operational taxonomy units (OTU) at a species level phytotype as well as 88-104 genera. Recognized were microbe taxa from *Firmicutes* but localized to *Streptococcus, Veillonella, and Granulicatella; Proteobacteria* phyla exhibited genera *Neisseria, Haemophilus; Actinobacteria* phyla showed genera *Corynbacteria, Rothia*, and *Actinomyces; Bacteriodes* phyla included *Prevotella, Capnocytopahga, Porphorymonas*, and *Fusobacteria* phyla with genus *Fusobacterium*. These finding are consistent with previous findings reported above (Ahn et al. 2012).
It is also notable that the cheek had the least diversity while the interproximal tooth samples had the highest diversity and this indicated that different anatomic oral sites with a difference in exposures to environment produced a variety of microorganisms present in the biofilm. Moreover, this study demonstrated a common core of bacteria that is present at each site. The specificity of this common core at the genus or phyla level does not appear to differ significantly when the 16S pyrosequencing approach is employed which is in contrast to findings reported using “healthy” and periodontal disease samples and other techniques such as disclosed by Mager et al. (2003), which uses a checkerboard DNA-DNA hybridization.

For example, more recent studies that used 16S pyrosequencing to detail the healthy oral microbiome demonstrated this common core of microbes at specific sites, such as the gingival sulcus. In a study by Abusleme et al. (2013) with an examination of the subgingival microbiome, they concluded that a disease state such as periodontal disease produced a process like an ecological succession “with emergence of newly dominant taxa in periodontal disease but without the replacement of primary health associated species”. Although, there is a high degree of inter-individual variability: “there was a core subgingival microbiome for this tissue”. It was also recognized that bleeding on probing did not change the subgingival microbe population. An important question this study alludes to is the presence of a similar core microbiome at other healthy oral tissue sites is also anticipated.

For example, there was recorded two OTUs closely related to Fusobacteria phyla and genus; Fusobacterium nucleatum. Moreover there were 11 other OTUs less frequently detected, which incorporated five genera of Actinomyces spp; and Streptococcus spp (e.g., S. sanguinis); two Proteobacteria phyla; a Bacteroides phyla member Porphyromonas spp.
derived microbes. An interesting finding was the maintenance of *Actinobacteria*, such as *Rothia spp.* and *Actinomyces spp.* in health and periodontal disease (Abusleme et al. 2013).

In another recent study, Camelo-Castillo et al. (2015) used a set of PCR primers amplified identification of 16S RNA accompanied by pyrosequencing of 22 sub gingival “healthy” samples but similarly identified as discussed above, these genera: Fusobacterium (phyla: Fusobacteria), Parvimonas (phyla: Firmicutes), Prevotella (phyla: Bacteroides), Porphyromonas (phyla:Bacteroides), Streptococcus (phyla: Firmicutes) and TM7 population of microbes. It is interesting to note that while recognizing representatives of phyla ascribed to the oral cavity microbiome noted above, the abundance percentages such as, subgingiva vary and this further emphasizes there is a high degree of inter-individual variability at the species and genus microbe level. This fact increases the difficulty to assess diversity of microbes at select sites.

Furthermore, similar to the previous study the most abundant genus in oral microbiome subgingival samples from all patients was the phyla Fusobacteria (e.g., 47% in “healthy subgingival samples) and the most abundant genera was Fusobacterium *nucleatum* in healthy subgingiva (Ge et al. 2013).

Taken together, these studies using subgingival samples are important because they 1) demonstrate a baseline for a healthy tissue, 2) show that a core microbiome is present, and also 3) demonstrate that diversity is influenced by anatomy of site, and microenvironment and we suggest these associations also occur at other sites in the oral cavity such as, the pharynx and tongue.
Another technique that attempts to provide a greater degree of taxonomic resolution is reported by Eren et al. (2014). In this study investigators used oligotyping of individual nucleotide positions that were information-rich sites because of their Shannon entropy identification. Shannon entropy provides an estimation of the average minimum number of nucleotide bases needed to identify a nucleotide sequence for a specific species of bacteria based on 493 oligotypes and their frequency in the Human Oral Microbiome Database from “healthy” individuals. The disadvantage to this approach is similar to that of 16S pyrosequencing which is a dependency on the number of “reads” that are used for analysis. In addition Shannon entropy provides information on variability to a greater degree of specificity and discrimination at a less than 1% difference between sequenced regions. This approach becomes essential because this technique does not use clustering as defined by OTU analysis so it is independent from taxonomy and more likely to find previously unknown taxa. For example, results showed a distinct and specific distribution of Streptococcus spp. (e.g., S.gordonii, S.salivaius) because Streptococccus spp. were not functionally redundant. In addition the investigators stated oral habitat groups are not randomly distributed as to taxonomy and oral genera but are specialized for specific microenvironments and there is a tendency for homogenization of the microbial species to a particular habitat. They further speculated there was a predominant regional anatomic effect for selection of specific microbe species that overrode local effects in “healthy” oral microbiome. Examination of close similarity of taxa oligotypes showed this effect for Neisseria spp, with N. flavescens/subflava composed nearly 100% of the Neisseria spp. on the dorsum of the tongue and keratinizing gingiva. In contrast a similar oligotype identified for N.sicca/mucosalflava but this Neisseria
spp. is found in plaque and keratinizing gingiva, buccal mucosa, saliva and hard palate but in lesser abundance.

Another interesting finding related to the distribution for specific microbe taxa stated above was the finding that “the majority of the microbial community” found in the palatal tonsil was also present in the hard palate, saliva, throat, and dorsum of the tongue but they also concluded that there was a “preferential” association of anerobes between the tonsil microbiome and the supra gingiva plaque microbiome (Eren et al. 2014).

iv. Age and Tissue Site and Oral Microflora Diversity

Compared to adult oral microbiome there is limited information regarding the child oral microbiome. However, Xu et al. (2014) evaluated 454 pyrosequences from 120 children at age 6 and used samples from intact enamel surfaces of 60 of these children. They disclosed the presence of five phyla and these were: Proteobacteria, Firmicutes, Actinobacteria, Fusobacteria, and Bacteroides which are also identified in adult oral microbiome. There were nine genera that were also identified and these were: Actinomyces (phyla: Actinobacteria) Capnocytophaga (phyla:Bacteroides) , Corynebacterium (phyla: Actinobacteria), Neisseria (phyla: Proteobacteria), Prevotella (phyla: Bacteroides), Streptococcus spp (phyla: Firmicutes), Veillonella (phyla: Firmicutes) and Derxia (phyla: Proteobacteria). They concluded this result demonstrated the presence of a core microbiome in children.

Vaginal delivery results in exposure of the newborn to vaginal microbial and systemic microbial contact. Colonization of the oral cavity begins within hours after birth and this is expected to be a result from exposure to mother, nurses, visitors, and hospital environment (MacFarlane et al. 2014). Above, we described the healthy oral microbiome of adults and the
observed effects on microbial diversity that was tied to the anatomic site and inter-individual variations as a consequence of exposures to living conditions, habits, and environment. In the oral cavity of newborns this process of selection and healthy homeostatic diversity is also documented at various oral mucosal surfaces and becomes further selective for microbial survival as dentition erupts into the oral cavity (Könönen 2000). Based upon culture and colony identification eight days after birth, both aerobes and anerobes are identified. The aerobic opportunistic and early colonizer; S. salivarius is detected eight hours after birth and in general Streptococcus spp. can represent 98% of the total oral microbiome until the eruption of dentition. For example, S.sanguinis colonization of infants is tooth-dependent and in competition with S.mutans (McCarthy et al. 1965; Smith et al. 1993). Another study used 51 healthy newborns (e.g., 10 min to 53h after birth) and a selective colony identification approach to evaluate samples from the tongue, buccal mucosa, alveolar process and palate. Nine samples did not produce growth, but 30.7% of the samples showed bacterial growth between 10 min and 8h after birth. For example, Staphylococcus aureus was identified in 37% of the samples which validated previous findings from Sliva et. al. who noted S. aureus in the mouth, anus, and nostrils of 49% of the newborns studied. It is assumed that S.aureus comes from exposure from mother or from attendants (Ross et al. 1980). However, the first microorganism identified in the oral cavity in 30.7% after birth was S. epidermidis, and this species increased to 90% after 93h of neonatal life. Furthermore, during the first year of life, Streptococcus (phyla: Firmicuts), Staphylococcus (phyla: Firmicuts), Neisseria (phyla: Proteobacteria), Lactobacilli (phyla: Firmicuts), Veillonella (phyla: Firmicuts), Actinomyces (phyla: Actinobacteria) and Fusobacterium (phyla: Fusobacteria). Notable were
levels of *Streptococci spp.* which found *Streptococci spp.* in 65.4% of the samples and *Staphylococcus spp.* was approximately found in 75.3% of the samples (McCarthy et al. 1965; Nelson-Filho et al. 2013; MacFarlane et al. 2014). Other samples from the first and second day of life contained *Streptococcus mitis* which persists until the end of the first year of life and *Streptococcus oralis* until three days after birth (Pearce et al. 1995).

Human oral microbiome for elderly populations has been largely unexplored. However, some studies have attempted to describe elderly microbiome but results are confounded by denture, hormones, long-term medication, diet and hygiene status. Moreover the methods used limited identification because a cultivation approach was used (Percival et al. 1991; Marsh et al. 2006). A study was undertaken by Preza et al. (2009) to evaluate the oral microbiome of 36 elderly (mean age of 84, 24 females and 6 males) at various sites. These included: dorsum of the tongue, (area of the tuberculum impar), buccal fold (mucosal area facing the first molar region of the fourth quadrant), hard palate (cross area between the palatine raphe and the second plica palatina transversa), supra-and subgingiva plaque from the same root surface. Evaluation was accomplished with Human Oral Microbial Identification Microarray (HOMIM) with 16A rRNA checkerboard hybridization. Combining all sites; eight phylogenetic groups were identified: *Firmicutes* (e.g., most common); and *Synergistetes* least likely detected phyla, while *TM7* and *Spirochaetales* were not detected at any site. Noted above in healthy adult oral microbiome *Firmicutus* members are the most common followed by *Proteobacteria* while *TM7*, *Spirochaetales*, and *Synergistetes* among the least common (Ahn et al. 2012). The general conclusion of this study was that the tongue and hard palate provided the lowest total number of bacteria species and there was a lack of
significant difference as to diversity between sites which are as listed both soft tissue and associated with the periodontum. Although the tongue had the highest number of significant association with the other sites and this was in contrast with supragingival plaque samples which had the lowest. In general species from *Eubacterium* (phyla: Firmicutes) and *Prevotella* (phyla: Bacteroidetes) were significantly associated with the tongue dorsum. Among species the highest prevalence for dorsum of the tongue found were: Veillonella *atypical*; Neisseria *flavescens*; *Streptococcus parasanguinis*, *S.salivarius*, and *S. parasanguinis*/BEO24.

In another study of tongue dorsum; reported by Riggio et al. (2008) identified microbes from scraping, PCR amplification, cloning and sequencing of 16S rRNA genes. They disclosed a difference in species between 12 control healthy subjects and 20 subjects with halitosis and elimination of other factors. Controls had: Lysobacter-type species; *Streptococcus salivarius*; *Rothia mucilaginosa*; *Veillonella dispar*; V. *atypical*; *Actinomyces odontolyticus*; *Atopobium parvulum*; and unidentified types. In contrast halitosis samples were composed of the following: Lysobacter-type species; *S. salivarius*; *Prevotella melaninogenica*; *P. veronella*; *P.pallens* and unidentified types which increased in number. This result shows that oral tissues can harbor a selective bacterial core in association with changes in physiology, biochemistry and molecular activity. Still another tongue study reported by Matsui et al. (2014) addressed the issue between microflora on the tongue and microflora in dental plaque to assess a crossover from one site to the other. This study focused upon the total bacteria and PCR based identification of *Fusobacterium nucleatum* which can be found at both sites. The results showed bacterial amounts on the tongue coating and in
dental plaque were independent of each other but a strong association between total bacteria and F.nucleatum from both sites existed.

Preza et al. (2009) showed that among elderly derived samples from buccal mucosa and hard palate there was a high prevalence of some species and these were: S. australis sp. strain T1-E5, and S. cristatus/BM053. Moreover, microbial diversity for identical sites from each individual is stated to “range considerably” and this means that in a comparison of oral sites there was a lack of common core or pattern. “Individuals with a low diversity at one site had more diversity at other sites and diversity tended to be independent of number of teeth, signs of gingivitis, pharmaceutical treatments, or place of residence”. Among the elderly; on a species level there were results for some correlation between microbiome and systemic disorders. These included H parainfluenzae oral taxon 718/ and A. arophilus 545 which were the most prevalent species at all sites and possibly associated with pneumonia (Sumi et al. 2006). In addition, Pseudomonas species were found on buccal mucosa and hard palate and these species are linked to tracheal and bronchial colonization or aspiration pneumonia (Niederman et al. 1989).

Changes in oral microbiome was also recognized to respond to changes in living environment. In a study by Takeshita T, et.al. they studied long-term hospitalized subjects (343 subjects, aged≥ 65) and the microflora of the dorsum of the tongue was shown to be colonized by Prevotella (phyla: Bacteroides ); Veillonella (phyla: Firmicutus); and Treponema (phyla: Spirochaetes) species as they developed pneumonia (Takeshita et al. 2010).
A comparison of these studies indicates the high degree of individual and cultural diversity and lack of a consistent core microflora which is an important finding as we attempt to correlate environment change related to tobacco smoke to status of oral microbiome and assessment a possible consistent disease pathogenesis at various inflammatory sites such as gingival periodontitis. In addition, comparison of oral microbiome differences between infant, child, adult or adult elderly oral microbiome as noted above demonstrates the enhanced susceptibility to robust environmental chemical exposures such as, tobacco smoke is expected to have on infant/child and elderly microbiome in contrast to adult because of the status of these microbiomes. For example, we note above the dynamic/development selective process that is evident in infant/child as compared to adult. This means that infant/children are likely candidates for effects from second hand tobacco smoke and other environmental chemical exposures in the form of inappropriate replacement of *Streptococcus* spp. and *Staphylococcus* spp. For children who have almost achieved the diversity of adult microbiome, the final phase of diversity is expected to be suppressed. The consequence of the loss of diversity at any age is to promote risk for infectious disease. In addition, the elderly oral microbiome is under selective stress because of increased susceptibility for systemic diseases that are also effected by depressed and stressed to reduce expected functional activity of immune activities.

Moreover, oral “healthy” microbiome undergoes rapid and significant changes in response to chemicals from the environment to effect metabolism (e.g. nitrosation; alcohol dehydrogenase); digestion (e.g., glucanase; peptidases); physiology (e.g., human papilloma virus infection) and oral mucosa viability (e.g., attachment to cemental enamel junction; desmosome/epithelial bridges and junctions) (He et al. 2015).
There are obvious connection to infection and loss of healthy oral microbiome but there is also growing evidence that systemic diseases not only among elderly populations discussed above but in children and adult populations. It is also thought that microbial biofilm quorum sensing and adaptive synergistic manner response to microenvironment changes results in increased risk for disease (Griffen et al. 2012; Hajishengallis et al. 2012). For example diabetes mellitus (Löe 1993); cardiovascular disease (Figuero et al. 2011); bacteremia (Bahrani-Mougeot et al. 2008).

There are many environmental-derived factors that contribute to a change in oral microbiome, and tobacco smoke exposure appears to have a profound effect on diversity and we suggest also metabolism to create a DNA damaging environment that further increases risk for infectious and non-infectious diseases such as cancer.

**B. Smoking**

About 45,000,000 people, or 20% of all those older than age 18, in the US are cigarette smokers. (CDC 2012). Furthermore, smoking is the the highest cause of preventable mortality in the US, (CDC 2002) attributing to more than 440,000 fatalities (25%) in the US each year (CDC 2010).

Smoking exerts its destructive effects to nearly every system, and is correlated with many pathologies, decreasing life expectancy and quality of life. According to the Report of the Surgeon General (2010), smoking-attributable diseases include lung cancer, cardiovascular disease, stroke, emphysema, bronchitis, and cancers of the oral cavity, bladder, kidney, stomach, liver, and cervix. Approximately half of long-term smokers
will die early on account of smoking, and those who die before the age of 70 will lose on average around 20 years of life (Doll et al. 1994).

Hecht et al. (1999) reviewed that tobacco smoke contains numerous deleterious compounds, including a gas phase and a particulate phase. In the gas phase exists carbon monoxide, ammonia, formaldehyde, hydrogen cyanide, and many other toxic and irritant compounds, including more than 60 known carcinogens such as dimethylnitrosamine and, importantly, the family of chemicals known as polycyclic aromatic hydrocarbons (PAH), including benzo(a)pyrene (Haritash et al. 2009). The particulate phase includes nicotine, “tar” (which itself is composed of toxic elements), benzene, and PAHs such as benzo(a)pyrene. Nicotine, an alkaloid, is found within the tobacco leaf and evaporates when the cigarette is lighted. It is quickly absorbed in the lung and crosses the blood-brain-barrier within ten to nineteen seconds (Benowitz 1998).

In addition, tobacco products have several associations to the microbiome that have not previously been considered. It is suggested in several reports that both bacteria and fungi colonize tobacco leaf and market product, which assumes an increase risk to alter the microbiome of tobacco users and this may be associated with a risk for enhanced toxicity as a result fore-mentioned metabolism (Hamilton et al. 1969).

All tobacco products are an agricultural product and naturally contains bacteria and fungi as nearly all types of crops. Forgacs et al. (1966) were able to identify that all tobacco products purchased on the open market all contained fungal mycelia, spores, and heat stable mycotoxins. Microbes found in tobacco via cultures, biochemical test, microscopy, or genetic tests include Acinetobacter, Bacillus, Burkholderia Closteridium, Klebsiella, Pseudomonas,
Serratia, Campylobacter, Enterococcus, Proteus, Staphylococcus, Pantoea and Aspergillus (Larsson et al. 2012). Moreover, it is recognized and documented in reports that tobacco leaf and tobacco products will contain risk for exposure to a set of microbes, which is at least 121 microbial species; that are derived from the soil, harvest or processing conditions associated with the growth of the tobacco plant. About 30% of these are reported to be linked to human diseases and metabolism of PAH and about 22% are harvested from oral cancer sites.

Relevant to microbe capacity to alter metabolism of host and microbe participants in a biofilm is presence of lipopolysaccharide, and mannose contained in cell walls, microbial derived peptides and peptidoglycans that can modify innate and adaptive host inflammatory reactivity; mucosa protection, and ultimately integrity. A mucosa architecture breakdown will enhances invasion of microorganisms into layers of oral mucosa keratinocyte structures (Devine 2003).

Tobacco smoke composition contains nearly 5000 chemicals with a variety of toxic, mutagenic, and carcinogenic activities that contributes to a survival selection of microbes based on degrees of sensitivity, and resistance to these chemicals. PAH metabolism by microbes generate reactive oxygen species that include: diol-epoxides, alcohols, and aldehydes (Rodgman et al. 2000; Vineis et al. 2000; Boström et al. 2002; Hecht 2003; Schick et al. 2005).

There are similar chemistries present in aquatic environments such as linked to degradation of petroleum products such as poly-cyclic aromatic hydrocarbons (PAH) that are regulated by microbes from select phyla and genera. Some of these simulate oral environmental niches at oral tissue sites as commensal microbes respond to exposure to

The identification of at these 55 genera of microbes indicates the expectation that there are 100’s of bacteria species that have this capability is an important consideration for the possible associations between microbes and host tissue risk for DNA damage through PAH metabolism.

### i. Periodontal Disease, Tobacco Smoke, and Microbial Biofilm Modification

As reviewed in Carranza’s text (2011), smoking is “the major risk factor for periodontitis,” increasing the prevalence, extent, and severity of disease. Moreover, smoking negatively influences the clinical outcome of non-surgical and surgical therapeutic regimens including the long-term success of implant placement. With 42% of periodontitis patients in the US attributed to smoking (Tomar et al. 2000), it is essential to understand its impact on initiation, progression, and management of the disease.

Tobacco smoke effect on oral tissues with emphasis on periodontal tissues is to decrease microbial diversity and reported are changes from a healthy microbial homeostatic, synergistic relationship between oral microbiome and oral mucosa to an enhanced state of infectious disease.
One example of this association is *Eubacterium nodatum*; a member of the complex of microbes identified by Haffajee and Socransky in subgingival samples from tobacco smokers, with a capacity to metabolize PAH and degradation (KEGG Pathway fnu00624) (Vu et al. 2009). Furthermore, other important genera for PAH bioremediation are *Bacteroides* and *Porphyromonadae spp.*

In this subgingival complex these genera are represented by the species members: *Prevotella intermedia* and *Porphyromonas gingivalis* (Haritash et al. 2009; Seo et al. 2009; Singh 2011). Moreover *P. gingivalis* is noted in KEGG microbial pathway, to have a methyltransferase activity to release 1-Methoxy-phenanthrene, 1-Methoxy-pyrene which will be further oxidized to 1-Methoxypyrene-6,7 oxide, and 1-Hydroxy-6-methoxypyrene and again through another methyltransferase produce 1,6 Dimethoxypyrene. It is important to note that *Pseudomonas spp.* members also have the capacity for metabolism of PAH chemicals from a variety of substrates such as Benzo[a]pyrene; pyrene; phenanthrene; anthracene, and fluorine through expression of genes nahB and doxE. *Pseudomonas aeruginosa* is also able to oxidize the methyl groups of the PAH, 7,12 dimethylbenzanthracene found in tobacco particulates to form hydroxymethyl derivatives (Haugen et al. 1986; McMillan et al. 1988; Denome et al. 1993)

*Peptostreptococcus micros* is another species identified with a high degree of presence in subgingival samples at periodontal sites from tobacco smokers and is recognized to cause a variety of human infections. *Peptostreptococcus spp.* can cause abscesses in the gingival tissues, as well as the brain, liver, breast, and lung. They contribute to in mixed infections with *Streptococcus* and *Staphylococcus spp.* *P. micros* is also found in respiratory and aero-
digestive and urinary infections but are only about 4% of all Peptostreptococcus species infections (Higaki et al. 2000).

This latter association between *P. micros* and other common oral infectious bacteria demonstrates the need to isolate selective species and to also characterize the microbe ecology in which the species of interest occurs. Further it is interesting to note that *Staphylococcus spp.* and some *Streptococcus spp.* can also metabolize PAH.

*Prevotella nigrescens* or *P. intermedia*, are members of the complex of bacteria detected in subgingival periodontal disease samples from cigarette users and another member of this genus, *P. ruminicola* is genetic close relative of the above bacteria species and although isolated from rumen samples this *Prevotella* species can metabolize and degrade PAH (KEGG Microbe pathway). It is also recognized that the phyla/order of *Bacteroidetes* are genetically close relatives of the genus *Prevotella* spp. and latter members of *Bacteroides* genus are well documented to metabolize and degrade PAH chemicals (Vinas et al. 2005).

Another bacterium species identified in periodontal disease subgingival samples from tobacco smokers is from the genus of *Bacillaceae* (e.g., *B. forsythus*). Members of this genus are commonly associated with PAH metabolism and degradation (Espinasse et al. 2003; Roh et al. 2007).

We anticipate a significant contribution by most of the identified genera found in periodontal subgingival infections of tobacco smokers to have a function in producing DNA damaging reactive oxygen substances (ROS). Moreover, release of ROS from metabolism and degradation of PAH, alcohols (e.g., acetaldehyde); volatile aromatic compounds (e.g.,
aldehydes, epoxides) results in a previous unrecognized reservoir of DNA damage and a source for loss of gingiva viability (e.g., loss of attachment to cemental enamel junction).

Acetaldehyde, is a carcinogenic by-product of tobacco and ethanol, that is shown to have a 2x increase in salivary production by oral microbes in patients with poor dental status (Homann et al. 2001). In addition, oral bacteria; *Streptococcus salivarius*, *Corynebacterium*, *Stomatococcus*, and alpha-hemo-lysing *Streptococcus* have been shown to have relatively high acetaldehyde production capability (Homann et al. 2000). Additional oral microorganisms have also been investigated for their potentially acetaldehyde synthesis and cancer-causing capabilities, includes *Candida albicans*. This fungus is found on the gingiva, palate, buccal mucosa, tongue and floor of the mouth of immunosuppressed, and immune compromised individuals (Hooper et al. 2009; Bakri et al. 2014; Alnuaimi et al. 2015; Bakri et al. 2015). *Candida spp.* is also isolated from leukoplakia lesions and these isolates can synthesize DNA damaging agents such as nitrosamines; exemplified by N-nitrosobenzylmethylamine (NBMA) which is recognized as a carcinogen (Krogh 1990; Barrett et al. 1998; Alnuaimi et al. 2015).

It is our expectation that these DNA damage releases will eventually lead to a loss of mucosa keratinocyte viability that accelerates microorganism access to the proliferative zone of oral mucosa. This process also will expose stem cell and transit amplified progeny populations to DNA damaging agents. Moreover a lack of critical faulty repair of DNA damage and inappropriate differentiation and maturations will lead to errors in oral growth regulation of keratinocytes and this will result in loss of mucosa integrity and risks for various oral mucosa diseases such as, periodontal, keratotic, epithelial dysplasias and cancers.
C. **Oral Cancer**

The Report of the Surgeon General (CDC 2010) reviews several mechanisms of smoking causing cancer. Carcinogens in cigarette smoke are metabolically activated by cytochrome P-450 enzymes, causing DNA adduct formation. In terms of PAHs, smoking also surges the incidence of DNA adducts from cigarette carcinogens such as benzo(a)pyrene and nitrosamines that are tobacco-specific. Examining proteins, smoke-related carcinogens lead to DNA damage with later mutations in “gatekeeper genes” (“tumor suppressor”) TP53 and (“oncogene”) KRAS. While this has been studied in lung cancer, this may be a mechanism in oral cancer.

Similarly, smoking has been found to lead to methylation of important “tumor suppressor” gene promoters such as P16. Examining epithelium, smoke components such as nicotine and nitrosamine “4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone” has shown stimulated signal transduction of pathways that allow the survival of impaired epithelial cells and resistance to apoptosis. Finally, cessation of smoking has been the sole verified approach to decrease the disease-causing processes that lead to cancer because the actual influence of many tobacco carcinogens to the development of cancer have still not been identified. However the reversal of diversity reduction in the oral microbiome is not reported and this feature of prolonged damage is expected to characterize a process of partial recovery (CDC 2010).

Oral cancer traditionally affects men in their fifties and sixties (Llewellyn et al. 2004). Although it is less prevalent in young adults with between 0.5-5% of cases occurring in those under 45 (Llewellyn et al. 2003), new evidence demonstrates that the
incidence in the below-45 age grouping is increasing (Chaturvedi et al. 2011) and is unique as to exposure to human papilloma virus subtype 16 (Van Monsjou et al. 2010; Pytynia et al. 2014).

Furthermore, recent studies have shown that the differential between male and female prevalence has decreased considerably, with significant increase in cases in women less than 45 years of age (Patel et al. 2011).

Several lifestyle habits are correlated with an increased risk of developing oral cancer, notably tobacco and alcohol use (Johnson 2001). Risk of OSCC due to the combination of tobacco with alcohol is approximately 80% or greater, with an up to 38x risk compared to those who do not use either substance (Blot et al. 1988). These two have been observed to have a synergistic effect (Pelucchi et al. 2008), with the effect of each being greater than the sum of their individual effects. The responsibility of either risk factor, however, is still not fully understood: oral squamous cell carcinoma is multifactorial, with no single recognizable cause being identified.

The use of tobacco products, as well as poor oral hygiene, for example, contribute to risk for development of these cancers (Homann et al. 2001; Meyer et al. 2008; Fitzpatrick et al. 2010).

Repeated exposures to tobacco smoke is also associated with increased risk a variety of diseases in the head and neck; such as, oral squamous cell carcinoma (OSCC) and upper airway infections.
i. Oral Microbiome and Cancer

Combined, this evidence suggests the hypothesis that tobacco smoke changes the content of the oral microbiome to result in loss of healthy homeostatic regulation and absence of disease. This process begins with the tobacco product and is amplified through metabolism of particular chemical families; such as, PAHs provided through tobacco smoke to create a chronic dysbiotic environment. We further suggest that dysbiosis creates more opportunities for loss of normal oral mucosa protection and a heighten level of inflammatory diseases.

Another class of chemicals often associated with risk for DNA damage is reactive oxygen species derived from nitrosation. For example nitrate reductase isolates from the tongue include genera from the phylum, Firmicutes: This include; Staphylococcus epidermidis; Veillonella atypica and dispar; phylum Actinobacteria; to include: Actinomyces odontolyticus; naeslundii; and genera; Rothia mucilaginosa, and dentocariosa. Presence of these microbes on the tongue or from bacterial colonies in dental plaque associated with supragingival or subgingival samples results in conversion of nitrate to nitrites and with acidification to nitric oxide, a reactive oxygen species is synthesized as a final product of de-nitrification (Doel et al. 2005; Korde et al. 2010; Pereira et al. 2015).

In OSCC there is a selective increase in presence of genera linked to particular ecologic microenvironments. However, the results obtained depend on the technique employed for identification. Studies that use culture provide different results from studies that use molecular methods that focused on a few phylum. Furthermore results from small numbers of clones per sample and sequenced provide a different array of bacteria (Nagy et al. 1998; Hooper et al. 2007). For example, culture of microbes representatives for Firmicutes
was *Veillonella* and *Streptococcus spps.*; Actinobacteria, *Actinomyces spp.* was shown to be increased in OSCC. In contrast, a high through-put genetic study based on microbiome 16S rDNA identification; a reduction in *Streptococcus spps.* and *Actinobacteria spps.* (e.g., Rothia mucilaginosa) in OSCC was reported. However, other phyla were represented in OSCC and these include; for example, *Bacteriodetes: Prevotella spp and Porphyromonas spp* and *Proteobacteria: Haemophilus spp* (Nagy et al. 1998; Hooper et al. 2007; Schmidt et al. 2014).

Noted above fungi such as, C. albicans contributes to a state of oral mucosal DNA damage. Furthermore, fungal contribution to development of OSCC is overlooked as fungal agents collaborate with selective microbe populations to further enhance risk for DNA damage. There are 80 known genera of fungi that are capable of PAH metabolism with a percentage identified to grow in the oral cavity; for example a variety of species of *Aspergillus* and *Candida spp* are reported to have this capacity. It is further interesting to note that fungal growth occurs in the presence of environmental factors such as tobacco smoke with increased levels of fungi, e.g., yeast and molds noted (Hattemer-Frey et al. 1991; Talhout et al. 2011). *Candida spp.* is highly prevalent in tobacco smokers with little change in biodiversity among fungal organisms in contrast to the relationship between smoking and a possible reduction in microbial biodiversity. It is difficult to document a clear assessment change in diversity among *Candida* genera for normal healthy non-smoking individuals. However, *Candida spp* is the most frequent genus identified followed by *Cladosporium spp.; Aureobasidium spp.;* and least detected compared is *Aspergillus* and *Cryptococcus spp* (Ghannoum et al. 2010; Monteiro-da-Silva et al. 2013; Monteiro-da-Silva et al. 2014).
It is notable that fungi such as Candida biotypes and others such as *Penicillium* and *Asperigillus* spp. have the capacity to metabolize PAH a family of chemicals derived from tobacco smoking (Boonchan et al. 2000; Zheng et al. 2003; Silva et al. 2009; Reyes-César et al. 2014).

*Candida* spp. is also isolated from leukoplakia lesions and these are expected to synthesize N-nitrosobenzylmethylamine (NBMA) which is recognized as a carcinogen. Stated above, Candida also synthesizes acetaldehyde a carcinogen associated with increased risk for OSCC and a recent report by Anuaimi et al., found an increase association with alcohol use and presence of OSCC and *Candida albicans* (Krogh 1990; O’Grady et al. 1992; Hooper et al. 2009; Bakri et al. 2014; Alnuaimi et al. 2015; Bakri et al. 2015). Still other fungi are reported to metabolize and degrade PAHs. (Field et al. 1992; Boonchan et al. 2000; Syed et al. 2011).

In addition to synthesis of DNA damaging chemicals, fungal and bacteria form a complex quorum sensing system. For example *C. albicans* and *Streptococci* spp. in the oral cavity or *C. albicans* and *Pseudomonas aeruginosa* interact with release of phenazines to enhance toxicity on to the oral mucosal surface. (Gibson et al. 2009; Peleg et al. 2010). In addition, Candida spp. release enzymes such as aspartic and proteases that can alter the architecture of the oral mucosa; and modify keratinocytes cell membrane with epithelial E-cadherins expression changes. These types of effects suggest an accelerated invasion of microbes with a loss of oral mucosa integrity which can enhance risk for DNA damage (Filler et al. 2006; Rouabhia et al. 2012; Rane et al. 2014; Staniszewska et al. 2014; Cavalcanti et al. 2015).

Lastly it is recognized that risk for OSCC through microbe metabolism is a product of genetic polymorphic expressions. OSCC patients that express genetic polymorphisms that
reflect in-appropriate inflammatory reactions to microbes, for example, NFkB1-294-ATTG; TNF308-A2A2/A2A1 and TNFb252-B2B2/B2B1 differ from healthy individuals that do not have these genetic polymorphisms. It is interesting to note that this group of genes is associated with lymphocyte, PMN, and macrophage responses to bacterial lipoproteins which have a bacteria or tobacco smoke origin. (Hasday et al. 1999; Barnes et al. 2007; Larsson et al. 2012; Brunotto et al. 2014) (Yapijakis et al. 2009; Lin et al. 2012).

Our testable hypothesis is an examination of oral microbiome at various tissues which include oropharynx (throat), tongue and gingiva will exhibit a change in diversity; and possible presence of selected genera that have a capacity for PAH metabolism as a result of exposure to tobacco smoke and presence of periodontal diseases.

There are two aims of the study:

1) The first is to determine a baseline number of bacteria genera present in throat and oral cavity for adults exposed to primary (mainstream) tobacco smoke or individuals that are not current tobacco users and are not exposed to secondary tobacco smoke.

2) Second is to demonstrate preferential and differential bacteria growth from current smokers in comparison to current non-smokers and never smokers following exposure to PAHs.

This approach is significant and is expected to provide novel insights into microbial effects on risk for oral cancer.
2. Methods

A. Patient selection

This study was approved by the Institutional Review Board of University of Illinois at Chicago (UIC 2011-0018) and was conducted in the Department of Periodontics, University of Illinois at Chicago College of Dentistry, Chicago, IL. N=24 patients were selected according to the following inclusion criteria: (1) generalized moderate- to severe- chronic periodontal disease (Armitage 1999), (2) smoking cigarettes for at least three years, (3) smoking at least 10 cigarettes per day (Martinez-Canut et al. 1995) and exclusion criteria: (1) Aggressive periodontitis, acute periodontal infection, perio-endo lesion, neoplastic/HPV lesion, (2) ASA Class III or above, (3) antibiotic use in past 3 months, (4) recently treated periodontitis.

B. Periodontal exam and diagnosis

Probing depths, recession, bleeding on probing, attachment loss, mobility and furcation involvement were recorded.

C. Sample collection

Subgingival plaque was obtained from teeth disease with periodontitis (AL ≥ 3 mm with inflammatory signs) and teeth that periodontally exhibited no signs of disease using paper points, put immediately into Tris buffer. Using a cotton swab, samples were additionally collected from the lateral tongue and oropharynx, put into Tris buffer.

D. Quantitation of 16 sRNA levels

Gram positive DNA purification kit “MGP04100” from Epicentre (Illumina) following standard protocol (Caporaso et al. 2010; Caporaso et al. 2012).
E. Conversion of 16sRNA quantitation to levels of taxonomic units

Sequence data were processed using the software package QIIME (“Quantitative Insights Into Microbial Ecology”) (Caporaso et al. 2010). Raw 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_libaries_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 numbers

For determination of differential representation of microbial species or genera in different sample sets we used STAMP. 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 (Parks et al. 2014).
3. Results

Comparisons between subjects with periodontitis who were smokers and non-smokers (Figures 1 and 2) were made, as well as between non-smoking subjects with and without periodontitis (Figure 3).

Figure 1: Significant differences comparing smokers to non-smokers on the tongue. A negative difference between means denotes a smaller presence of bacteria in smokers.

A significant decrease (p<0.05) in bacteria from the phyla actinobacteria, bacteroidetes, firmicutes, fusobacteria, planctomycetes, proteobacteria, and TM7 were detected on the tongues of smokers. No organisms were found to increase in relative abundance.
Figure 2: Significant differences comparing smokers to non-smokers in the oropharynx. A negative difference between means denotes a smaller presence of bacteria in smokers. A significant decrease (p<0.05) in bacteria from the phyla actinobacteria, bacteriodetes, firmicutes, fusobacteria, planctomycetes, proteobacteria, and TM7 were detected in the oropharynx of smokers. No organisms were found to increase in relative abundance.
Figure 3: Significant differences comparing non-smokers with periodontitis to non-smokers without periodontitis. A negative difference between means denotes a smaller presence of bacteria in non-smokers without periodontitis.

A significant decrease (p<0.05) in bacteria from the phyla actinobacteria, bacteriodetes, fircmutes, fusobacteria, proteobacteria, spirochaetes, synergistetes, tenericutes, and TM7 were detected in subjects without periodontitis. No organisms were found to increase in relative abundance in health, indicating a greater overall amount of bacterial diversity in periodontitis.
4. Discussion

A strong trend for a decrease in microbial diversity was noted in smokers, while an increase in diversity was found in subjects with periodontitis. This has been found in other 16s rRNA studies published recently. Bizzarro et al. (2013) found less diversity in subgingival samples of smokers, with this in turn affecting the severity of periodontal disease: “…lower taxonomic diversity showed on average higher periodontal attachment loss.” Accordingly, Camelo-Castillo et al. (2015) noted a less diverse oral microbiota in subgingival samples. In contrast, however, Charlson et al. (2010) found that microbiota from smokers were actually more diverse than non-smokers in the oropharynx, and Bizzarro et al. (2013) found no differences using both culture and PCR methods.

Relating to periodontitis, Griffen et al. (2012) found the lowest Shannon diversity value for health, with a dose-dependent increase in shallow and then deeper periodontal pockets. Similarly, Abusleme et al. (2013) noted increased diversity in disease. Following this, Camelo-Castillo et al. (2015) noted that the number of species (as determined using OTUs at 97%) were highest in subjects with periodontitis. In contrast to these results, an earlier study using the DNA-DNA checkerboard approach (Mager et al. 2003) found no statistically significant changes on oral mucous membranes of subjects with periodontitis.

In addition to the effects of smoking and periodontitis on quantitatively measured diversity, a qualitative examination of the altered microbiota suggests an increase in dysbiosis for both conditions. For example, Novosphingobium spp., which were increased in periodontitis, are known to degrade PAH and are also associated with the unburnt tobacco leaf (Sapkota 2010). Additionally, Rutebemberwa et. al. (2014) noted that N. aromaticivorans, and
*N. capsulatum* are PAH degraders (Demaneche et al. 2004; Sohn et al. 2004; Rutebemberwa et al. 2014).

Bacteroidetes were also found in our study to be increased in periodontitis, and an increased abundance of bacteroidetes, including *Prevotella tannerae* were found in precancerous and OSCC lesions compared to samples from healthy normal individuals (Schmidt et al. 2014). Similarly, Preza et al. (2009) reported that *Prevotella spp.* are often found in OSCC lesions, and Vinas et al. (2005) noted that bacteroides spp. can metabolize PAH.

Additionally, *Rothia spp.* were noted in our study to increase in periodontitis. Abusleme et al. (2013) reported that the presence of these microbes results in the conversion of nitrate to nitrites and, with the acidification to nitric oxide, a reactive oxygen species. The authors also stated that Rohis is the most common genera associated with OSCC lesions.

Another species increased in our study is *Staphylococcus epidermidis*. Pushalkart et. al (2011) noted that nitrate reductase isolates from the tongue included this species, and also that some *Staphylococcus spp.* have the capability to metabolize PAH. Additionally, (Schmidt et al. 2014) found the firmicutes phylum (including *Staphylococcus*) to be strongly associated with OSCC.

Summarizing dysbiosis as relating to the presence of smoking and periodontitis, (Ahn et al. 2012) reviewed that periodontal microorganisms may activate cigarette- associated carcinogens in the local environment or perhaps globally through chronic inflammation: “Establishing the association of the oral microbiome with cancer risk may lead to significant advances in understanding of cancer etiology, potentially opening a new research paradigm for cancer prevention.” Additionally, in a case-control study of 105 men by Tezal et al.
(2007), after adjusting for confounders, every mm of crestal bone loss was associated with a 5x risk of oral cancers. Although no mechanism was given, this study shows that these associations have been previously reported in the literature.

This study is significant because it proves that diversity is increased in periodontitis and decreased with smoking, and additionally suggests a novel mechanism between these changes in the microbiota and the risk for oral cancers. In the future, it will be important to validate these findings with more subjects, especially increasing the number of smokers. This is also a cross-sectional study, which determines only association, not causality.
5. Works Cited


6. Vita

Name Harrison Edward Mackler

Education
B.A., Biology, Clark University, Worcester, MA 2007
D.M.D., Harvard School of Dental Medicine, Boston, MA, 2012
Periodontics Certificate, University of Illinois at Chicago, Chicago, IL, 2015

Professional Membership
American Academy of Periodontology, Midwest Society of Periodontics,
Illinois Society of Periodontists

Publications
