IMPACT OF PERIODONTAL INTERVENTION ON LOCAL INFLAMMATION, PERIODONTAL DISEASE AND HIV OUTCOMES

Justin R. Valentine

A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the School of Dentistry (Periodontology)

Chapel Hill
2016

Approved by:
Jennifer Webster-Cyriaque
Janet Southerland
Thiago Morelli
ABSTRACT

JUSTIN R. VALENTINE: Impact of Periodontal Intervention on Local Inflammation, Periodontal Disease and HIV Outcomes
(Under the direction of Jennifer Webster-Cyriaque, Janet Southerland, and Thiago Morelli)

Forty-six percent of the United States population is infected with periodontitis, and of these, 37.1 and 8.9% suffer from severe and mild to moderate forms of the disease, respectively. 1.2 million persons in the US are also diagnosed with HIV. Increasing evidence suggests that the chronic periodontal infection is implicated in the generation of a systemic inflammatory response, which represents a potential risk factor for worsening various systemic conditions, including HIV. It has been reported in the literature that gut microbial translocation can lead to immune activation of HIV. There are few if any studies investigating the potential impact of microbial translocation of the oral cavity on HIV and, importantly, the impact that the resolution of oral inflammation has on HIV immune status.

The purpose of this study was to identify periodontal disease status and disease resolution following periodontal intervention, and assess the therapeutic response of local inflammation and HIV outcomes, as measured by pro-inflammatory cytokine IL-6, CD4 cell count and HIV viral load in a cohort of HIV positive patients.
ACKNOWLEDGEMENTS

I wish to acknowledge my mentor, Jennifer Webster-Cyriaque, DDS, PHD for her caring, patience, wisdom and encouragement that has guided me to the completion of this project. We have met on many after-hour coffee appointments, typing, writing, searching, editing and theorizing the concepts of this important project. I thank her for the opportunity to partake in this intellectual journey, as it has made me a better scientist and better clinician.

My most sincere gratitude to my thesis committee members, Janet Southerland DDS, PHD and Dr. Thiago Morelli DDS, MS for their invaluable contributions in editing and conceptual suggestions. I am especially gracious to Thatsanee Saladyanant DDS, PHD for her help with data analysis, Jo-Ann Blake, RDH for periodontal chartings, Kathy Ramsey for data management and Jannet Doolittle literature review contributions.

Lastly, I want to thank my family, Mom, Dad and brother, who have emotionally, financially and spiritually supported me throughout my journey.
# TABLE OF CONTENTS

LIST OF TABLES .................................................................................................................. vi

LIST OF FIGURES .................................................................................................................. vii

LIST OF ABBREVIATIONS ...................................................................................................... viii

CHAPTER 1: THE EVIDENCE OF BACTERIA, INFLAMMATION AND VIRUSES IN PERIODONTAL DISEASE PATHOGENESIS

Introduction .............................................................................................................................. 1

Section 1.1 Periodontal disease .............................................................................................. 2

Section 1.2 Bacterial etiology of periodontal disease .............................................................. 3

Section 1.3 Periodontal disease and inflammation ................................................................. 7

Section 1.4 Periodontal disease in the setting of immunosuppression ................................. 11

Section 1.5 Viruses and periodontal disease ......................................................................... 13

Section 1.6 Potential mechanisms of viral and bacterial co-infections ............................... 15

   KSHV ................................................................................................................................. 15

   EBV ................................................................................................................................. 17

   HPV ................................................................................................................................. 18

Summary ............................................................................................................................... 19

CHAPTER 2: IMPACT OF PERIODONTAL INTERVENTION ON LOCAL INFLAMMATION, PERIODONTAL DISEASE AND HIV OUTCOMES ........................................... 21

Introduction ........................................................................................................................... 21

Section 2.1 Methods and materials ...................................................................................... 24

   Setting ............................................................................................................................... 24
LIST OF TABLES

Table
1. Baseline demographic profiles of study population..........................................................27
2. Study schema for study population from baseline to 24 months.................................26
3. Periodontal disease classification of study population baseline to 12 months..............28
4. Median log HIV VL, CD4 count and log IL-6 stratified by BGI.................................33
5. BGI changes in the long-term suppressed group baseline to 12 months........................33
LIST OF FIGURES

Figure

1. Percentage category change for classification systems from baseline to 12 months........29
2. Demographics stratified by BGI and age adjusted probabilities for mod/severe disease...31
3. ART and HIV VL status for subjects followed for 12(a) and 24(b) months..................32
4. Changes in HIV VL(a) and CD4(b) from baseline to 12 months based on ART status…..35
5. Age-adjusted probabilities for mod/severe periodontitis stratified by [IL-6](a), [IL-6] stratified by BGI(b), [IL-6] stratified by ART status..................................................36
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP</td>
<td>American Academy of Periodontology</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>BGI</td>
<td>Biofilm Gingival Interface</td>
</tr>
<tr>
<td>BGIG</td>
<td>Biofilm gingival interface</td>
</tr>
<tr>
<td>BGIH</td>
<td>Biofilm Gingival Interface Healthy</td>
</tr>
<tr>
<td>BOP</td>
<td>Bleeding on probing</td>
</tr>
<tr>
<td>CAL</td>
<td>Clinical attachment loss</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FN</td>
<td><em>Fusobacterium nucleatum</em></td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>HDACi</td>
<td>Histone de-acetylase inhibitor</td>
</tr>
<tr>
<td>HHV</td>
<td>Human herpes virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s sarcoma herpes virus</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LGE</td>
<td>Linear gingival erythema</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>NUG</td>
<td>Necrotizing ulcerative gingivitis</td>
</tr>
<tr>
<td>NUP</td>
<td>Necrotizing ulcerative periodontitis</td>
</tr>
<tr>
<td>NUS</td>
<td>Necrotizing ulcerative stomatitis</td>
</tr>
<tr>
<td>OMV</td>
<td>outer membrane vehicle</td>
</tr>
<tr>
<td>P1</td>
<td>Periodontitis mild</td>
</tr>
<tr>
<td>P2</td>
<td>Periodontitis moderate</td>
</tr>
<tr>
<td>P3</td>
<td>Periodontitis severe</td>
</tr>
<tr>
<td>PG</td>
<td><em>Porphyromonas gingivalis</em></td>
</tr>
<tr>
<td>PD</td>
<td>Probing depth</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis System</td>
</tr>
<tr>
<td>SCFA</td>
<td>Small chain fatty acid</td>
</tr>
<tr>
<td>SEQ</td>
<td>Sequence</td>
</tr>
<tr>
<td>SPNS</td>
<td>Special Projects of National Significance</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Tumor necrosis factor- alpha</td>
</tr>
<tr>
<td>UNC</td>
<td>University of North Carolina</td>
</tr>
<tr>
<td>VL</td>
<td>Viral load</td>
</tr>
</tbody>
</table>
CHAPTER 1: THE EVIDENCE OF BACTERIA, INFLAMMATION AND VIRUSES IN PERIODONTAL DISEASE PATHOGENESIS

INTRODUCTION

Polymicrobial infections are central to the pathogenesis of many diseases. These infections incite the host immune system and the potential for pathogen-pathogen interactions in this setting may worsen disease. Oral infections of the periodontium are characterized by multiple bacterial agents, and more recently, viral infections as well. Traditionally, periodontal disease has been characterized as an inflammatory disease caused by bacteria found in dental plaque and the host immune response.

This review serves to discuss periodontal disease and its associated host responses, the potential molecular mechanisms that arise during viral and bacterial co-infections, the role of viruses in periodontal disease and periodontal disease in the setting of systemic viral infection and immune suppression.

Section 1.1 Periodontal Disease

Periodontal disease is an inflammatory disease that may lead to the loss of supporting bone, cementum and periodontal ligament, and could ultimately lead to exfoliation of the teeth. The disease is broadly categorized into two forms: gingivitis, which affects the gingival tissues surrounding the teeth; and periodontitis, which affects the gingival tissues, bone, cementum and periodontal ligament surrounding the teeth. Periodontitis is always preceded by gingivitis; however, not all gingivitis lesions will progress to periodontitis. Over the course of time, various
concepts of the etiology of periodontal disease have and continue to evolve. What follows is a brief historical overview of the etiology of periodontal disease.

Periodontal disease has been documented since antiquity. Earliest known documentations are from Ancient Egyptian Hieratic writings. Dating back to approximately 3700 BC, Ebers papyrus from Egyptian documents detailed some of the early practices of medicine. In these documents, there are references to periodontal disease, described as “throbbing blisters” in teeth, likely periodontal abscesses. It was also documented that various surgical treatments led to “strengthening of the flesh” or gums. Other ancient cultures would also record similar observations of periodontal disease, including India, China and Greece. During this time period, periodontal disease, as well as most diseases, was thought to originate from divine intervention and retribution of sinful acts. Thus, treatment was often associated with religion, through various rituals and prayers.

During the Arabic period of medical history (622AD-1492), newer concepts and treatments in medicine evolved. In the 18th century, dentistry began separating from medicine and developed into its own profession and concepts of periodontal disease started to evolve. Such theories were published in *The Surgeon Dentist* by Pierre Fauchard, 1728. Fauchard is regarded as the father of modern dentistry and described periodontal disease as a local phenomenon of the oral cavity characterized by inflamed, edematous gums often with purulence, and associated these findings with poor oral hygiene. What was unknown at the time was what entity caused the disease. Fauchard was correct in his assessment of the link between oral debris and periodontal disease. But it would not be until the discovery of bacteria by Van Leuwenhoek in 17th century that the composition of this debris would be characterized and set the stage for the
role of bacteria in periodontal disease. William Younger was one of the first dentists to suggest that periodontal disease was of bacterial etiology in his work *Pyorrhea Alveolaris*, 1905.

Currently, periodontal disease is believed to involve primarily two factors: bacteria and the host response, which are influenced by genetics and modified by various risk factors, such as smoking and diabetes. Although studies have shown strong associations between bacteria and periodontal disease, bacterial alone do not fully explain the expression of the disease. As Offenbacher stated, “bacteria are necessary but not sufficient” for periodontal disease. The objectives of this review are to summarize the evidence of bacteria as a causative agent for periodontal disease, the roles of inflammation and viruses in periodontal disease and the association between HIV and periodontal disease.

**Section 1.2 Bacterial etiology of Periodontal Disease**

The evidence for a bacterial etiology in periodontal disease is well described in the periodontal classical literature. One of the most classic studies showing a clinical relationship between bacteria and gingivitis was performed by Loe, et al. In this study, twelve subjects with good oral hygiene abstained from oral hygiene practices until the development of clinical gingivitis. Microbial plaque samples were taken and analyzed with light microscopy from the start of the study until the expression of gingivitis, at which time oral hygiene practices were reinstituted. All subjects accumulated large amounts of plaque and developed gingivitis. Microbial changes were characterized by a change from predominately coccal forms of bacteria at the start of the experiment, to filamentous and spirochetal forms during the later part of the experiment, when gingivitis was induced. Upon reinstition of oral hygiene practices, gingival health was reestablished in all subjects, and in the majority of subjects, the microbiota returned to predominately cocci and short rods, while a minority (17%) of subjects still presented with
filamentous bacteria. This classic study demonstrates that in healthy subjects, the occurrence of gingivitis was concomitant with the occurrence of increased amounts of microbial plaque and changes in the microbial composition of the plaque. Furthermore, removal of the microbial plaque led to resolution of the disease, with concomitant return to cocci and short rod forms in the majority of subjects.

Listgarten investigated the structural changes in the microbiota associated with teeth in subjects who were periodontally healthy and those with varying severities of periodontal disease, including periodontitis, periodontosis and post-periodontitis, the latter two entities representing what is currently named aggressive periodontitis. The difference between periodontosis and post-periodontitis is separated by age, where the former was in subjects ≤21 years of age and the later for subjects ≥22 years of age. It was observed that the morphological features of the microbiota were significantly different amongst the groups.

Healthy subjects had bacterial cells predominately coccoid shaped, gram-positive organisms. Filamentous and gram-negative organisms were low in number compared to other subject groups. Gingivitis samples consisted of densely packed microbial cells associated with the tooth surface coronal to the epithelial attachment. The bacterial cells were coccoid and filamentous, some flagellated with both gram-positive and gram-negative patterns observed. The filamentous bacteria were more numerous and a distinctive “corn-cob” bacterial formation pattern was observed in some areas. Periodontitis samples consisted of predominantly filamentous forms, along with the cell types and patterns identified in gingivitis samples. There were a substantially increased number of flagellated bacteria and the deeper pocket layers demonstrated “test-tube brush” configurations. Periodontosis samples contained gram-negative coccoid and filamentous bacteria and lacked grossly noticeable surface deposits while post-periodontosis
samples consisted of flora similar to gingivitis and periodontitis supragingivally, and periodontitis in the apical portions.

From Listgarten’s study, one can appreciate how the complexity of microorganisms increases with advancing disease severity. As the periodontal pocket increases in depth, it creates an environment conducive to the proliferation of more pathogenic microorganisms. As an example, many periodontal pathogens are obligate anaerobic organisms. As the depth of the pathological pocket and quantity of bacteria increases, the oxygen content of the pocket decreases. This can often be observed clinically as cyanosis of the gingival tissues in areas of severe gingival inflammation. Furthermore, gingival crevicular fluid, cellular debris, proteins, and hemorrhage increase, all of which serve as a nutrient source for bacteria. The biofilm formation of the microbiota, as observed by the increase in structural complexity between bacteria, allows for communication, sharing of nutrient resources and evasion of host immune response and systemic antibiotic effects.

A study of subgingival microflora and periodontal disease, Slots recruited subjects who had gingivitis, advanced adult periodontitis or juvenile periodontitis, while healthy subjects served as controls. Subgingival microbial plaque was sampled from the periodontal pocket using sterile currettes and bacteria analyzed using anaerobic jar culturing. It was observed that the microbiota differed between subject groups. In healthy individuals, gram-positive bacteria dominated the sample, representing 85% of cultivable organisms, primarily Streptococcus and Actinomyces species. Gingivitis, adult and juvenile periodontitis had higher amounts of gram negative organisms, including *F. nucleatum, B. melaninogenicus*, and *Bacteroides* species.  

Socransky identified and grouped oral bacteria into specific complexes based on cluster analyses. Using 185 subjects, sub-gingival bacterial plaque samples were collected from
patients with periodontitis (n=160) and without (n=25). From a total of 13,000 plaque samples, checkerboard DNA-DNA hybridization was performed for 40 bacterial taxa. Statistical clustering analyses revealed 5 major sub-gingival bacterial complexes: red, orange, green, yellow, and purple.

The red and orange complexes have been associated with periodontitis, while the green complex has been associated with health. For example, Socransky showed that species of the red and orange complex had a significantly positive association with deep probing depths. Pocket depths were their shallowest when red complex bacteria were absent and deepest when in the presence of all red complex bacteria. Other studies have identified red complex organisms in association with periodontitis.\textsuperscript{7,8} More recent investigations have further corroborated these results.\textsuperscript{9–15} Virulence factors have been identified for pathogenic periodontal bacteria. These virulence factors are related to surface components for attachment (pili, long fibers, capsules, vesicles), evasion of the host immune system (leukotoxin, chemtaxis inhibitors, lymphocyte alterations, immunoglobulin proteases, fibrinolysis, superoxide dismutase), enzymes (collagenase, acid phosphatase) and toxins (LPS).\textsuperscript{16}

More recently, the field of metagenomics has allowed the detailed description of bacterial community composition. Previously, such descriptions relied on culture methods, leaving a multitude of bacteria in biological samples unidentified. This is because the oral cavity harbors many fastidious bacteria that are difficult to isolate via culture methods. Now, with high-throughput sequencing techniques, such as 454 pyrosequencing, investigators are rapidly identifying new species associated with periodontal disease and health. Using 454 pyrosequencing, for example, Griffen, et al. sequenced bacteria in subgingival plaque samples from 29 individuals who had periodontal disease and 29 individuals who were healthy.\textsuperscript{17}
species were identified and unique, statistically significant differences in species prevalence were described between disease and healthy subjects. Interestingly, many of these species are uncultivable and are not described in the classic bacteria-complex model developed by Socransky⁶.

**Section 1.3 Periodontal disease and inflammation**

Inflammation is frequently viewed as a double-edged sword. On the one end, there is activation of the host’s immune system, the transition from innate to the adaptive immune response and the clearing of the immunologic challenge, such as bacteria or a foreign body. On the other end there is collateral damage that is often viewed as a negative consequence of the inflammatory process.

However, one should consider that the destructive process serves as a protective function to the host. For example, in pregnant women, a septic infection in the mother triggers a large increase in TNF-a, which may stimulate the spontaneous delivery of the fetus to protect her from the infection in the mother. In periodontal disease, bacterial infections around teeth activate macrophages and fibroblasts to secrete inflammatory cytokines, which in-turn leads activation of osteoclasts and degradation of connective tissue and bone surrounding the teeth.

As an end-result, this process places the host’s vital tissues, connective tissue and bone, at a greater distance from the bacteria and bacterial toxins, such as LPS and leukotoxins. This allows for epithelium down-growth and the creation of a wider buffer between noxious stimuli and the host’s vital tissues. In extreme cases, the entire tooth may exfoliate, which is again protective to the host, since now the portal for bacterial infection and progression has been eliminated. Nonetheless, the loss of connective tissue and bone from inflammation results in periodontitis. This process begins with gingivitis.
In the healthy periodontium, inflammatory cells, primarily neutrophils, are present in the gingival sulcus as a result of the presence of normal bacterial flora near the gingival sulcus. However, there is an equilibrium in existence here, whereby the amount and interactions of bacteria and inflammatory cells present do not lead to the irreversible destruction of the attachment apparatus of the tooth: connective tissue and bone. But, as the microbial challenge increases, there is increased collagen breakdown of the gingival tissues resulting from the stimulated release matrix metalloproteinases (MMPs) from macrophage and fibroblast cells. Clinically this manifests as edematous, red or cyanotic tissues that bleed easily on gentle pressure. This reversible, plaque-induced gingival lesion is known as gingivitis.

Classic studies by Loe et al in the 1970s examined the natural progression of plaque induced gingival lesions in Sri Lankan tea laborers over a 15 year period. It was the first study to assess tooth loss as a function of time using a group of tea laborers who performed no oral hygiene practices. The findings were that all individuals harbored large amounts of plaque and gingival inflammation around teeth. The progression of attachment loss around teeth and tooth mortality was assessed and 3 unique groups were identified based their rates of periodontal disease progression: rapid (8%), moderate (81%), and no progression (11%). Although most individuals lost periodontal attachment over time, some subjects never lost attachment despite large amounts of plaque and gingival inflammation. Thus the progression of periodontal disease is not the same for all individuals, nor is the loss of attachment a linear function.

Prior to the 1980s, periodontal attachment loss was believed to be of a slow and continuous progression. However, many longitudinal human and animal studies have shown that some periodontal sites lose attachment at rates faster and slower than what would be expected from a continuous model process. For example, radiographic analyses of periodontal bone...
levels assessed longitudinally for 10 years revealed rates of bone loss inconsistent with a 
continuous model\textsuperscript{21}. Socransky demonstrated in a cohort of 64 adults with no periodontal 
treatment over a 6 year period that individuals experienced an annual attachment loss of 0.18mm 
per site per subject; however, only 12\% of all sites examined experienced attachment loss over 
2mm, which would be considered significant. Furthermore, of those sites experiencing >2mm of 
attachment loss in the first 3 years of the study, only 40\% would progress further over the last 3 
years of the study\textsuperscript{22}. Similar findings were also demonstrated in animal studies.\textsuperscript{23}

Socransky, et al studied and proposed a burst model for the rate of periodontal attachment 
loss\textsuperscript{22}. He hypothesized that periodontal disease activity may be explained by a burst or 
asynchronous burst model, whereby individuals experience disease activity in random bursts 
with regard to time and history of previous attachment loss. Furthermore, these burst episodes 
may occur more frequently during certain periods of life, and subsequently become quiescent 
asynchronous burst model). Although there is no proof that the burst hypothesis is true, it does 
help explain the shortcomings of the continuous model.

In a review by Page, et al,\textsuperscript{24} the major “players” of the inflammatory-driven, periodontal 
destruction were identified and include the junctional epithelial cells, fibroblasts, leukocytes and 
cytokines- each player playing her part in the drama of inflammation. There seems to be 
equilibrium at play between inflammatory cytokines and anabolic growth factors, between 
periodontal destruction and periodontal repair and regeneration. Indeed, this is reflected in the 
classic study mentioned previously by Loe et al\textsuperscript{3}. In that study, most individuals progressed to 
more severe forms of periodontitis; however, about 10\% of individuals never progressed beyond 
gingivitis.
Therefore, balance between gingival inflammation and connective tissue destruction and extracellular matrix synthesis allowed for the disease to never progress beyond gingivitis. Animal and human studies on periodontitis have shown different time periods of disease activity balanced by disease quiescence and even repair\textsuperscript{19,20,22,23,25}. In fact, such is reflected in Socransky’s random burst models. Periodontal disease is not a linear, continuous function, but at certain points, anabolic growth factors and periodontal repair supersede cytokine-mediated destruction, which clinically is reflected in no further progression of attachment loss and in some instances, even attachment gain is detected.

Gingivitis can be divided into three separate stages: initial, early and established\textsuperscript{26}. The initial lesion exhibits signs of acute inflammation with increased gingival fluid flow, enhanced neutrophil diapedesis, exudation and deposition of fibrin in the affected area. Collagen of the marginal gingiva may be destroyed in some areas. The early lesion exhibits an infiltrate of lymphocytes and macrophages with small amounts of plasma cells. Lymphocytes comprise the majority of cells, at roughly 75%. Vasculitis and large quantities of neutrophils in the junctional epithelium are observed. Resident fibroblasts undergo pathological changes and lymphocytes become activated. During the early phase, clinical signs of gingivitis may be present. The established phase consists histologically with large quantities of B-cells and plasma cells, in addition to T-cells. Large quantities of neutrophils in the sulcular epithelium are surrounded by macrophages in the lamina propria and lymphocytes in the connective tissue. Progression beyond the established lesion is the advanced lesion, which leads to periodontitis. Interestingly, in the established lesion there appears to be two types: those that remain stable and do not progress and those that progress and lead to periodontal destructive lesions.
There are 5 main innate protective mechanisms at play: neutrophils, epithelium, saliva, gingival crevicular fluid, high level of tissue turnover. Neutrophils are recruited from the systemic circulation into the gingival tissues, where they migrate towards the microbial plaque and chemo-attractants such as IL-8 secreted by epithelial cells. Over 500,000 neutrophils are estimated to migrate into the oral cavity per minute, in a normally functioning host\textsuperscript{27}. Thus, the neutrophil serves as the first barrier to the underlying connective tissue and bone. Epithelial barriers include oral epithelium, oral sulcular epithelium, and junctional epithelium. The oral epithelium covers the underlying connective tissue and bone and is contiguous with the oral sulcular epithelium, which lines the sulcus, faces the tooth and continues to the junctional epithelium. Junctional epithelium separates the sulcus environment from the underlying connective tissue and bone. It is approximately 30 cell layers thick in its coronal aspect and tapers to approximately 3 cell layers in its most apical aspect\textsuperscript{28}. Saliva has several important protective properties, including its flushing action, presence of secretory IgA, salivary agglutinins and presence of leukocytes. Gingival crevicular fluid is a serum transudate and contains many protective components including complement proteins, which are activated in inflammation and aid in phagocytosis. Lastly, high cell turnover rate is also protective, for as inflammatory-mediated host tissue destruction occurs, concurrently, anabolic host tissue synthesis occurs, creating an equilibrium that, in a healthy state, does not lead to attachment loss.

The events that occur from established to advanced lesion include the microbial stimulation, via virulence factors such as LPS, of pro-inflammatory cells such as macrophages, to release of inflammatory cytokines IL-1, TNF-a, and PGE-2. T-lymphocytes also become activated and release IL-1 and lymphotoxin, which can stimulate macrophages and fibroblasts to produce matrix metallo-proteinases (MMP), which break down collagen in the extra-cellular
matrix. Other cytokines help to regulate or reverse the response to the pro-inflammatory actions. For instance, transforming growth factor-β (TGF-β) suppresses production of MMPs, and IL-1 receptor antagonist competitively binds to IL-1 receptor but exhibits no pro-inflammatory effect.

Section 1.4 Periodontal Disease in the setting of Immunesuppression

It has been documented that individuals with HIV have a higher prevalence of periodontitis than individuals who are HIV negative, with some studies showing prevalence as high as 85%. Furthermore, HIV positive individuals may express unique forms of periodontal disease. Referred to as HIV associated periodontal disease, these conditions include linear gingival erythema (LGE), necrotizing ulcerative gingivitis (NUG), necrotizing ulcerative periodontitis (NUP) and necrotizing ulcerative stomatitis (NUS).

There is also a higher prevalence of oral fungal infections, including oral candidiasis, aspergillosis, cryptococcosis, histoplasmosis, blastomycosis, paracoccidiodomycosis, and zygomycosis. Although with the availability of highly active anti-retroviral therapy (HAART) the prevalence of the HIV associated periodontal diseases has declined globally, they still remain a concern for patients and health care practitioners who treat these lesions when they occur.

In the context of immunosuppression, one must consider the importance of the potential for opportunistic infections to influence the severity of periodontal disease. Given the establishment of the important role of not only bacteria, but also viruses and fungi in periodontal disease, investigating the differences of the microbial flora between HIV positive and HIV negative individuals is important. In a comparison study of 8 periodontal putative pathogens in chronic periodontitis patients, significant differences in subgingival microbiota were detected between HIV positive and negative individuals. These same investigators also noted that the
periodontal pathogen, *P. nigresens*, was statistically associated with an increased odds for the presence of periodontal pathogens *P. gingivalis, C. rectus, and T. denticola*. In a Brazilian study performing a similar comparison, Goncalves, et al. reported that putative periodontal pathogens were found in greater numbers in HIV negative patients with chronic periodontitis than HIV positive patients. However, the HIV positive patients exhibited statistically significantly higher amounts of *E. faecalis* and *A. baumannii*, bacteria that are not usually associated with periodontal disease. Similar observations were made by other investigators.

However, some studies have shown no difference between the subgingival microbiota of HIV positive and negative individuals. For example, Murray, et al. observed similar periodontal pathogens in HIV positive chronic periodontitis individuals that is typically found in HIV negative chronic periodontitis patients. The investigator did find, however, that HIV associated gingivitis lesions consisted of a microbiota different from that commonly seen in HIV negative individuals. Other studies have shown both the presence of typical periodontal pathogens along with atypical microorganisms that may play a role in the progression and severity of periodontal disease.

Periodontal pathogens may also stimulate HIV reactivation in latently infected immune cells. Imai, et al. demonstrated *in vitro* that butyric acid-producing bacteria can increase HIV reactivation as detected by p24 antigen assay. Supernatant from periodontal pathogens *F. nucleatum* and *P. gingivalis* substantially induced HIV-1 replication, even beyond that of positive assay controls. A different group, Huang, et al. demonstrated similar findings. Using HIV-infected T-cell, macrophage and dendritic cell lines, periodontal pathogens, including *P. gingivalis, F. nucleatum, A. actinomycetemcomitans, C. rectus, and T. forsythia* were shown to increase the promoter activation for HIV replication by as much as 34 fold in certain cell lines.
**Section 1.5 Viruses and Periodontal disease**

Clinical studies that have assessed the relationship between viruses and periodontal disease have consistently identified the human herpes viruses (HHV) in diseased samples. There are 8 HHVs known to man. Those that have been most commonly associated with periodontal disease include the Herpes Simplex Virus (HSV), Human Cytomegalo Virus (HCMV), and Epstein Barr Virus (EBV). Association studies have compared the prevalence of these HHVs between patients with periodontitis and gingivitis/shallow pocket sites.\textsuperscript{44-50}

When comparing HSV, EBV and HCMV detection in gingival biopsies of periodontally healthy vs. chronic periodontitis, Contreras et al. showed statistically significantly greater counts of HHVs in periodontal disease vs. health.\textsuperscript{44} Ting, et al. compared the prevalence of HHVs in deep vs. shallow sites of localized aggressive periodontitis patients and found that deeper, more diseased periodontal sites harbored a greater number of detectable HHVs than shallow, less diseased sites.\textsuperscript{51} In his review of viruses in periodontal disease, Cappuyns, et al. summarized the various association studies of Slots’ group and showed that in general, more diseased periodontal sites harbored a greater number of detectable HHVs than shallow, less diseased sites.\textsuperscript{52}

In a group of 10 subjects, Das, et al. collected subgingival plaque samples from patients with chronic and aggressive periodontitis and demonstrated a statistically significant associations between HSV-1 and EBV and periodontal disease.\textsuperscript{45} Whereas Das et al. utilized multiplex PCR for detection of virus, Kubar, et al. used real-time polymerase chain reaction (RT-PCR) to both detect and quantify HHVs in patients with chronic and aggressive periodontitis. Sites with the highest quantity of virus were detected which had the deepest probing depths.\textsuperscript{49} These studies are of great clinical relevance, as patients most afflicted by aggressive periodontitis tend to be adolescents and young adults and they experience a rapid loss of periodontal attachment, often in
the absence of plaque and calculus. Just as chronic periodontitis has been thought of as primarily a bacterial etiology without much consideration to viruses, so has the aggressive form of periodontitis. Increasing our knowledge and understanding of this relatively rare form of disease will enhance our abilities to treat it.

The overall conclusion from these groups of studies is that HHVs are statistically associated with periodontal diseases, including gingivitis, localized and generalized chronic and aggressive periodontitis.

**Section 1.6 Potential mechanisms of viral and bacterial co-infections**

Recent studies have begun to elucidate molecular mechanisms by which bacteria could exacerbate viral pathogenesis in the oral cavity. The red complex bacterium *Porphyromonas gingivalis* and orange complex bacterium *Fusobacterium nucleatum* are widely studied in connection with periodontal disease and are Gram-negative, anaerobic, rod-shaped bacteria. *P. gingivalis* and *F. nucleatum* secrete a number of metabolic end products, including LPS, gingipains, outer membrane vehicles (OMVs), and short chain fatty acids (SCFAs) such as butyric acid$^{53-58}$. Butyric acid levels can be up to 20 mM within the gingival pocket$^{56,59,60}$. SCFAs like butyric acid are known histone deactylase inhibitors (HDACi)$^{58}$ that may induce acetylation of histones within neighboring cells, leading to reactivation of latent viruses$^{56,61-63}$. Molecular signaling pathways induced by SCFAs or other bacterial products may also contribute to epigenetic re-modeling or other mechanisms of viral induction.

**KSHV**

Kaposi’s Sarcoma Herpes Virus (KSHV) exhibits latent infection or active lytic infection, two distinct phases of replication, both of which are essential for long-term persistence of herpesviruses. Latent KSHV has been shown to be reactivated, or induced to enter the lytic phase
of replication, by spent media containing metabolic components from periodontal pathogens. In BCBL-1 cells with latent KSHV, reactivation has been observed following exposure to *P. gingivalis* or *F. nucleatum* spent media by a number of different assays, including transcription of the late lytic gene *K8.1*, production of RTA, v-Ii6, and K8.1 proteins, Gardella gel showing production of linear viral genomes, presence of DNase-resistant DNA indicating virion production, and infectivity of these virions by observing production of LANA, RTA, and K8.1 in A293T cells infected the supernatant. Together, these assays demonstrate the full lytic cycle can be completed after exposure to *P. gingivalis* and *F. nucleatum* metabolic products. Further, only certain bacterial species have the ability to reactivate KSHV, with spent media from *P. intermedia*, *S. aureus*, and *S. mutans* failing to reactive KSHV.

Epigenetics play a critical role in regulation of KSHV gene expression. Several studies have shown that a chemical agent, sodium butyrate (NaB), and other HDAC inhibitors increase acetylation of histones regulating of gene expression and lead to KSHV reactivation. *P. gingivalis* and *F. nucleatum* spent media also have HDAC inhibition properties, as seen by a fluorimetric-based HDAC activity assay, that can increase global acetylation levels of H3 and H4. Following treatment with NaB or *P. gingivalis* or *F. nucleatum* spent media, increased histone acetylation was seen at the KSHV ORF50 as well as globally across the human genome. In addition to inhibition of HDAC activity, SCFAs made by *P. gingivalis* and *F. nucleatum* decease expression of EZH2 and SUV39H1, two enzymes that repress transcription by mediating histone methylation, specifically H3K27me3 and H3K9me3. Bacterial spent media leads to increases in activating acetyl marks and decreases in repressive methyl marks, facilitating expression of KSHV.
Additional cellular pathways are modulated by *P. gingivalis* spent media and potentially involved in viral reactivation. Hypoxia, induced by increased reactive oxygen species, pro-inflammatory cytokines, or by direct addition of hydrogen peroxide, is known to reactivate KSHV in BCBL-1 cells\textsuperscript{75,76}. The hypoxia response has also been implicated in reactivation induced by *P. gingivalis* spent media by microarray studies in BCBL-1 cells showing marked induction of this pathway and confirmed by a hypoxia response element luciferase reporter assay. Further, a hypoxia-responsive kinase, PIM-1, showed increased protein levels in *P. gingivalis* spent medium treated cells. PIM-1 is required for KSHV reactivation by another chemical, TPA, and has been shown to phosphorylate KSHV LANA, the protein primarily responsible for maintaining latency. Phosphorylation of LANA by PIM-1 disrupts two functions of LANA, repression of transcription from the KSHV terminal repeat and repression RTA autoactivation, promoting viral reactivation\textsuperscript{77}. There was no evidence of a hypoxia response to NaB treatment.

In addition to the hypoxia response pathway, the p38 MAPK pathway is also involved in bacterium-mediated induction of the KSHV lytic cycle. The p38 inhibitors SB202190 and PD169316 significantly reduced *P. gingivalis*-mediated KSHV reactivation, including production of v-IL6 and K8.1 proteins\textsuperscript{62}.

**EBV**

Spent media from *P. gingivalis* and *F. nucleatum* also induces lytic reactivation of another herpesvirus, EBV. In cells latently infected with EBV, *P. gingivalis* spent media induced expression of the BZLF1 gene product, ZEBRA, a transactivator for EBV lytic genes. This reactivation was least partially mediated by HDAC inhibition, attributed to butyric acid. Although *P. gingivalis* also produces isobutyric, propionic, acetic, and valeric acid, only butyric
acid treatment led to acetylation of H3, a mark of active chromatin, and induction of ZEBRA. ZEBRA expression also required structural remodeling of nucleosomal chromatin, as treatment with the topoisomerase II inhibitor novobiocin prevented \textit{P. gingivalis} induced ZEBRA production\textsuperscript{63,78}.

\textbf{HPV}

Unlike herpesviruses, HPV does not exhibit latent and lytic replication states. However, viral gene expression during the HPV lifecycle is tightly controlled and linked to epithelial differentiation\textsuperscript{79}. High risk HPVs cause cervical, oropharyngeal, and other epithelial cancers, largely due to expression of the viral oncogenes E6 and E7, which repress p53 and Rb\textsuperscript{80,81}. Recent studies have linked increased incidence of HPV+ oropharyngeal squamous cell carcinoma with chronic inflammation including periodontal disease\textsuperscript{82}. Indeed, a recent study has observed increased gene expression of E6 and E7 and increased protein expression of E7 in HPV16+ SiHa cells in cells treated with \textit{P. gingivalis} and \textit{F. nucleatum} spent media for 6 or 24 hrs. RNA-seq confirmed the increase in viral gene expression following exposure to bacterial spent media and dysregulation of cellular pathways involved in cancer pathogenesis and implicated in studies of bacterial-mediated modulation of other viruses, such as metabolism, invasion, the MAPK pathway, epigenetic regulation, and hypoxia response. \textit{P. gingivalis} spent media induced reactive oxygen species production in SiHa cells and induced the hypoxia response, as confirmed by hypoxia response element luciferase reporter assay. In addition, the activating marks H3ac, H3K9ac, H3K27ac, and H3K36me3 were increased globally in SiHa and specifically on the HPV long control region following \textit{P. gingivalis} spent media treatment. Increased HCK, ERK, and p38 phosphorylation were also observed, indicating dysregulation of cellular kinase pathways. Bacterial-mediated epigenetic changes and viral oncogene expression
were abrogated by SB202190 and PD169316 p38 inhibitors. Periodontal bacteria may contribute to oral carcinogenesis by promoting HPV oncogene expression by epigenetic remodeling, partially mediated by kinase signaling.

**Section 1.7 Summary**

The concepts of periodontal disease have evolved with the advent of better technologies to understand them. Since the Middle Ages, it has been understood that there was an association with poor oral hygiene, calculus and periodontal disease. In the 19th century the disease was conceptualized as autoimmune or degenerative, but in the 20th century that changed to one primarily involving bacteria and host response, and now the 21st century is recognizing the possible role of viruses in periodontal disease.

There is ample evidence to support the role of bacteria as a causative agent for periodontal disease. Yet one must consider that the classical studies by Loe, Listgarten, Slots, Socransky, etc., often utilized culture and light microscopic methods to “see what’s there” in periodontally diseased sites. Today we know that there are a multitude of uncultivable bacteria in periodontal sites that may have a role just as significant as the red complex pathogens.

Another uncultivable entity, outside of an infected host cell, is viruses. As obligate intracellular organisms, the only way to culture viruses are with living cells. The classical techniques used in the 70s and 80s would have missed the presence of viruses in periodontally diseased sites as growth media specific for bacteria was used. In the late 90s, researchers began investigating a possible association with herpes viruses and periodontal disease. The majority of these studies come primarily from one group of investigators, belonging to Slots’ group. This group was able to show statistically significant associations between various types of periodontal diseases and the presence of herpes viruses HSV, EBV and HCMV.
During immunosuppression, the nature of periodontal disease is different. Unique gingival lesions such as LGE, NUG, NUP and NUS may involve the presence of viruses, fungi and bacteria, and include many species not typically seen in an immunocompetent individual. Many studies have confirmed the presence of such atypical organisms in periodontally diseased sites,\textsuperscript{34–37} which may explain the reason that these individuals as a group have a higher prevalence of periodontitis.\textsuperscript{30,31}

Understanding that there is a significant association with herpes viruses and periodontal disease, and that there is also an undeniable role of bacteria in periodontal disease, one must consider if there is an interaction between viruses and bacteria during periodontal infection and whether this interaction can explain disease and disease progression. Studies have shown that bacterial metabolic end-products from periodonto-pathogenic organisms \textit{P. gingivalis} (PG) and \textit{F. nucleatum} (FN) may lead epigenetic modification of histones in herpes virus KSHV, which reactivates the virus to enter its lytic cycle of replication. Likewise, PG and FN have been shown \textit{in vitro} to induce reactivation of EBV. Given the association between herpes viruses and the various forms periodontal disease, understanding the interaction between virus and bacteria is of great significance and needs further investigation.
CHAPTER 2: IMPACT OF PERIODONTAL INTERVENTION ON LOCAL INFLAMMATION, PERIODONTAL DISEASE AND HIV OUTCOMES

Introduction

Oral lesions associated with human immunodeficiency virus (HIV) include oral candidiasis, Kaposi’s sarcoma, lymphoma and hairy leukoplakia. During the era of antiretroviral therapy (ART), the prevalence of oral human papillomavirus (HPV) and HIV-associated salivary gland disease has risen. While HIV/AIDS-associated periodontal lesions including linear gingival erythema and necrotizing ulcerative periodontitis are not as prevalent as in the pre-ART era, chronic periodontitis remains an issue in the HIV/AIDS population.

Along with many other systemic diseases, HIV/AIDS has been documented to have impact on the periodontium. Periodontitis is characterized by the host immune reaction to periodontal pathogenic microorganisms. While largely identified as an inflammatory disease caused by bacteria and bacterial by-products detected in dental plaque, there is growing evidence that viral infections are involved in periodontal disease. This inflammatory process is characterized by destruction of the attachment apparatus surrounding the teeth. The 2009–2012 National Health and Nutrition Examination Survey (NHANES) estimates that 46% (64.7 million) of the US population has periodontitis, of which 8.9 and 37.1% suffer from severe and mild to moderate forms of the disease, respectively. Increasing evidence suggests that the chronic periodontal infection is implicated in the generation of a systemic inflammatory response, which represents a potential risk factor for worsening various systemic conditions, including atherosclerosis, stroke, diabetes, and others.
Given its prevalence and impact, the detection and monitoring of periodontal disease progression is important. Chronic periodontal disease has been categorized using different classification systems. The most widely used classification system is based on clinical attachment levels, as described by Armitage and adopted by the American Academy of Periodontology (AAP)\(^8^9\). For the purposes of population surveillance, the Centers for Disease Control and Prevention developed a case classification in conjunction with the American Academy of Periodontology (CDC/AAP) based on periodontal probing depths and clinical attachment levels\(^9^0\). Lastly, the biofilm–gingival interface (BGI), a classification system based on bleeding on probing and probing depths, has been described\(^9^1\). The BGI classification reflects current periodontal status rather than historical levels of disease activity. Collectively, these systems may provide a better understanding of historic chronic disease, current status, and active disease and progression.

The systemic impact of HIV is multifaceted. Broad depletion of immune function allows susceptibility to opportunistic infections\(^9^2\). Further, systemic immune activation occurs secondary to microbial translocation. The degree of immune activation is strongly correlated with disease progression, morbidity, and mortality\(^9^3\)–\(^9^7\). Furthermore, decreased production of protective interleukins (IL), such as IL-6, IL-17, and IL-22, affects the epithelial tight junctions throughout the GI tract\(^9^7\) allowing for escape of immune-activating bacterial products into the systemic circulation. While immune activation-related microbial translocation from the gut has been well documented, the oral cavity may also serve as an important source of microbial translocation.

The mouth may harbor over 700 different bacterial species, billions of bacteria, and a multitude of viruses and fungi. Teeth are transmucosal, with crowns exposed to the contaminated
oral cavity and root structures secured within maxillary and mandibular bone. The two environments are separated by a junctional epithelium that functions as a barrier to prevent the penetration of bacteria and bacterial products to underlying connective tissue and bone. During chronic periodontal disease, the junctional epithelia seal is widened, leading to the translocation of bacteria and bacteria by-products to the underlying connective tissue and bone. Thus, chronic periodontal disease possesses the potential for oral microbial translocation as a source of systemic inflammation. This study aimed to determine the impact of periodontal treatment on local inflammation and systemic HIV status in a population of HIV-positive individuals.

Section 2.1 Methods and Materials

Setting

This group was part of a prospective multicenter longitudinal study for Special Projects of the National Significance (SPNS) Oral Demonstration Project. 196 subjects were enrolled in the multicenter study. A cohort of 73 HIV-positive subjects recruited to the UNC site was identified for the present study and analysis. Approval was obtained from the Institutional Review Boards of the University of North Carolina at Chapel Hill (UNC) and the Evaluation Center for HIV and Oral Health at Boston University. Subjects were examined and treated at the UNC Hospital Dental Clinic. The multisite centers maintained human subjects’ approval for the overall and individual studies.

Study design

At baseline, subjects received a comprehensive examination and treatment plan. Subjects were seen at least every 6 months for dental prophylaxis/debridement, and information was
collected including periodontal metrics: probing depths, bleeding on probing, and clinical attachment level. Baseline, 12- and 24-month assessments were included in this analysis. At these time points, whole un-stimulated saliva was collected. Of the 73 study participants, only 45 completed the study to the 24-month visit. Comprehensive dental care for the 73 participants included dental prophylaxis at least every 6 months, scaling and root planing, oral hygiene instruction, extractions, restorative and prosthetic dental treatment.

Additionally, a baseline interview was conducted that collected the following data: sociodemographic characteristics, mode of HIV transmission, past substance and tobacco, barriers to accessing oral health care since testing HIV positive, and oral healthcare habits. Interviews were conducted in both English and Spanish, and all participants gave informed consent to participate. Baseline data collection occurred from January 2008 to August 2011. Interviewers participated in a standardized training module. Interview and sample data were entered into a web-based database hosted by the multisite coordinating center, where the data were merged into a single multisite database. All examiners performing oral examinations and measures were calibrated to a gold standard with kappa score >0.9. CD4 counts and HIV viral load were recorded. Periodontal measures were also obtained and recorded. In a subset of 26 subjects, saliva samples were collected for the measurement of pro-inflammatory cytokines.

Participants

Subjects who aged 18 and above, who were HIV positive, and who were out of dental care for at least 12 months were eligible. Subjects were referred from infectious disease specialists and community healthcare centers.
Outcome variable

The outcome of significance for the current analysis was periodontal status pre- and post-intervention. Using the American Academy of Periodontology (AAP) and Centers for Disease Control and Prevention/AAP (CDC/AAP) and biofilm–gingival interface (BGI) classifications, participants were classified based on periodontal disease severity at baseline, 12 and 24 months. Changes in HIV viral load, CD4 and IL-6 levels and the relationships between demographics, HIV viral load, and concentration of IL-6 were also evaluated in the analyses.

Predictor variable

Periodontal status at baseline, and the exposure is the periodontal intervention. Potential confounders and effect modifiers included antiretroviral therapy and time on antiviral therapy.

Data sources

Information for medical status including CD4 and HIV viral load (HIV VL) was abstracted from the patient’s medical chart. Periodontal disease classification was determined from the periodontal examination. Enzyme-linked immunosorbent assay (ELISA) was used to measure pro-inflammatory cytokine IL-6 levels (QuantiKine Kit; R&D systems). Whole unstimulated saliva was collected and assayed in duplicate following the manufacturer’s instructions. Concentrations of the mediator were determined by optical density at the manufacturer’s recommended wavelength using a microplate reader (Epoch microplate spectrophotometer; Biotek). Duplicate readings were averaged, and the values were multiplied by the dilution factor.
Study size

The study size was comprised of a convenience sample of 73 subjects. Table 2 displays the study schema.

Table 2: Study Schema for UNC HIV Oral Demonstration Project Description of all treatments and information collected at baseline and every six months for 73 subjects over 12 months and 45 subjects over 24 months

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Baseline</th>
<th>6 Months</th>
<th>12 Months</th>
<th>18 Months</th>
<th>24 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interview</td>
<td>Prophy</td>
<td>Debridement</td>
<td>OHI</td>
<td>CD4/HIV VL</td>
<td>Periodontal charting</td>
</tr>
<tr>
<td></td>
<td>Prophy</td>
<td>Debridement</td>
<td>OHI</td>
<td>CD4/HIV VL</td>
<td>Periodontal charting</td>
</tr>
</tbody>
</table>

Section 2.2 Statistical analysis

Statistical analysis was accomplished through SAS/STAT, version 9.3 (SAS Institute Inc. 2011. STAT SAS 9.3 Procedures Guide, Cary, NC, USA: SAS Institute Inc.). Shifts in periodontal disease categories from baseline to 12 months were assessed separately by classification method using Bowker’s test of symmetry. For CD4, VL, and salivary IL-6, data were analyzed for subjects who were consecutively available for baseline and 12-, or baseline and 24-month appointments. Data points were non-parametrically distributed as determined by q-q plots. The changes in CD4, VL, and salivary IL-6 were compared across the 4 therapy groups none (not on ART at baseline), short (on ART for less than one year at baseline), long suppressed (on ART for >1 year at baseline with undetectable HIV viral loads), long not suppressed (on ART for >1 year at baseline with detectable HIV viral loads) using Kruskal–Wallis analysis followed by Mann–Whitney pairwise comparisons between therapy groups. Wilcoxon matched pairs signed rank analysis was used to compare baseline and 12-month and
baseline and 24-month values for CD4, VL, and IL-6 separately for each therapy group. Binary logistic regression analysis was used to determine age-adjusted probabilities for moderate/severe periodontitis based on CDC/AAP classification using STATA statistical software. Level of significance was set at 0.05 for all analyses.

Section 2.3 Results

Participants

A total of 73 subjects enrolled and were confirmed eligible in the UNC HIV Demonstration Project from 2008 to 2011; 73 were followed from baseline to 12 months, and 45 participants were followed from baseline to 24 months. All underwent consecutive periodontal measurements every six months. There were 54 men and 19 women (Table 1).

<table>
<thead>
<tr>
<th>Table 1 HIV-infected subjects’ demographic profile HIV (2008-2011) at baseline. Percent of each group is shown based on A) gender, B) race/ethnicity, C) income, D) smoking status, and E) age distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Gender cohort %</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>CD4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Smoker cohort %</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Income</td>
</tr>
<tr>
<td>Race/Ethnicity cohort %</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Overall, the mean age of participants was 44; the majority of participants were non-Hispanic (NH), Blacks (43), and Whites (24). For a full description of population demo-
graphics, see Table 1. Of individuals, 45% were smokers. At baseline, the overall mean CD4 and HIV viral load (VL) were 517 cells/ml and 72 043 copies/ml, respectively. At baseline, demographic values were collected and comprehensive oral examination with treatment planning was performed. Prophylaxis/debridement was carried out at baseline, 6, 12, 18, and 24 months (Table 2.) At baseline, 12 and 24 months, CD4 and HIV VL were obtained and whole un-stimulated saliva was collected for the measurement of IL-6. For the 73 participants over the course of the study, there were 293 dental prophylaxis/periodontal maintenance procedures, 19 scaling and root planing procedures, 33 periodontal debridements, 127 dental extractions, and 325 dental restorations placed.

Dental intervention in HIV-positive subjects resulted in periodontal disease resolution. Seventy-three HIV-positive subjects were classified according to AAP, CDC/AAP, and BGI periodontal disease classification systems (Table 3. and Figure 1).
AAP Classification- AAP measures periodontal disease based on clinical attachment levels, reflecting past disease status. The AAP classification at both baseline and post-intervention categorized 87.6% of subjects as having mild periodontitis at both baseline and post-intervention at 12 months. Pre- and post-intervention differences were not statistically significant for the AAP classification (P = 0.38, extended McNemar test). Of subjects, 4% improved by 1 category and 6.8% decreased by 1 category. The perceived worsened disease status was likely due to gingival recession associated with disease resolution.

AAP/CDC classification- AAP/CDC measures periodontal disease based on clinical attachment levels and probing depth based on a subset of sites, and this metric estimates disease. Using the AAP/CDC classification, 51% were classified in the moderate periodontitis group at baseline. At 12 months post-treatment, 13.7% of subjects improved by one category and 20.5% of subjects improved by 2 categories. The inclusion of periodontal pocket depths provides a measure of current disease status. However, pre- and post-intervention differences in classification were not statistically significant for the AAP/CDC classification (P = 0.255, extended McNemar test).

BGI Classification- BGI measures periodontal disease based on bleeding on probing (BOP) values and probing depths, and this metric considers inflammation (BOP). The biologically based classification, BGI, determined that 63% of the subjects had severe disease at baseline. Post- treatment at 12 months, only 1 subject remained in the severe disease category, with increasing number of subjects categorized less severe categories. At 12 months post-treatment, 56.2% of subjects improved by one category, 9.6% of subjects improved by two categories, and 9.6% of subjects improved by three categories. Thus, 54 patients showed improvement and pre- and post-intervention differences in classification were highly statistically
significant for the BGI classification (P < 0.0001, extended McNemar test). The improvement was likely due to the resolution of gingival inflammation. Compared to the AAP classification, the CDC/AAP and BGI classifications reflected current active disease and periodontal disease resolution over time (Figure 1).

In HIV-infected subjects, differences in age, smoking, and income were not associated with more severe periodontal disease BGI case classifications were used to assess the frequency of periodontal health, gingivitis, and disease in the context of demographic variables. High prevalence of severe periodontal disease in the HIV cohort was detected across all income levels and across all ages (Figure 2). An equivalent number of smokers and non-smokers had severe periodontal disease. Severe periodontal disease was detected in 70% of HIV-positive NH Blacks and 50% NH Whites. At baseline, severe periodontal disease was detected in 63% of men and in 63% of women with HIV infection. Similar percentages of men and women were detected in the moderate disease category.
Figure 2 (a) Differences in age, smoking, and income were not associated with the development of severe periodontal disease by BGI stratification in the context of HIV infection. Stratification of UNC HIV cohort demographic data: gender, race/ethnicity, income, smoking status, and age by BGI including periodontal health, gingivitis, mild periodontitis (P1), moderate periodontitis (P2), and severe periodontitis (P3). (b) The likelihood of developing moderate/severe periodontal disease was higher for the HIV group for all demographic risk factors. Age-adjusted predicted probabilities (95% CI) of moderate/severe periodontitis using CDC/AAP case classifications derived from multivariable binary logistic regression models. Estimates are for men and women; four age groups; major racial/ethnic groups (non-Hispanic White, non-Hispanic Black, Hispanic, Other); categories of annual family income ($’000); and smoking status.
Binary logistic regression analysis determined the likelihood of moderate/severe periodontal disease based on CDC/AAP classification (Figure 2). Moderate/severe periodontitis was positively associated with White and non-White races, smoking and non-smoking, male and female gender, all income levels, and all age groups. The probability of disease development was over 50% for both women and men and for both smokers and non-smokers. The probability of disease development was over 60% across all age groups and across all income levels. The probability of disease development was at least 50% for Whites and over 60% for Blacks and Hispanics.

ART and HIV status were associated with severe periodontal disease Of the 73 individuals followed longitudinally for one year, at baseline 63 were on ART and 10 were not. Of those subjects on ART, 51 were on ART for at least 12 months at baseline. Of these individuals, 28 demonstrated undetectable viral load (Figure 3a). There were 45 individuals followed for 24 months, of whom 39 were on ART for at least 12 months at baseline. Of these individuals, 25 demonstrated viral suppression with undetectable viral load at baseline (Figure 3b).
Regardless of ART status, over 80% of subjects had moderate/severe disease as determined by BGI classification (n = 73). BGI classification detected severe periodontal disease in 55% of those with undetectable viral loads (n = 28) and 75% of subjects on short-term ART (n = 12) Table 4. Interestingly, McNemar analysis did not detect statistically significant improvement in BGI classification using tests in the absence of ART (n = 10, P = 0.63), on short-term ART (n = 12, P = 0.53), or on long-term ART who were not suppressed (n = 21, P = 0.09). This lack of statistical association may be related to the small size of the groups. However, in those on long-term ART who were suppressed, a statistically significant improvement in BGI was detected (n = 28, P = 0.026) Table 5b. At least fifty percent of subjects in each of the four groups demonstrated at least one category of periodontal improvement (Table 5a).

Table 4 HIV and BGI periodontal status at baseline. Median log HIV VL, median CD4 cells ml⁻¹, median log IL-6 pg ml⁻¹, and frequency of subjects in each BGI category (healthy, gingivitis, mild, moderate, and severe periodontal disease) were provided for subjects not on ART, on short-term ART, on long-term ART, on long-term ART who were virologically suppressed and who were not virologically suppressed.

<table>
<thead>
<tr>
<th></th>
<th>No art N = 10</th>
<th>Long term ART (&gt;12 months) Suppressed N = 28</th>
<th>Long term ART (&gt;12 months) Not suppressed N = 23</th>
<th>Short term ART &lt; 12 months N = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Log VL</td>
<td>10.24</td>
<td>Undetectable</td>
<td>5.7</td>
<td>8.5</td>
</tr>
<tr>
<td>Median CD4</td>
<td>568</td>
<td>469</td>
<td>437</td>
<td>468</td>
</tr>
<tr>
<td>Median log salivary IL-6</td>
<td>1.08</td>
<td>3.8</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Healthy</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
<td>10</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Severe</td>
<td>7</td>
<td>15</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>28</td>
<td>23</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 5 BGI changed over time with the intervention (a) Difference in BGI classification overtime based on ART status, (b) BGI changes in the long-term ART-suppressed group (n = 28, P = 0.028)

<table>
<thead>
<tr>
<th>Category change</th>
<th>-1</th>
<th>-2</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ART</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Short term ART</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Long term ART suppressed</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>15</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Long term ART not Suppressed</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>13</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>1</td>
<td>15</td>
<td>39</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b)</th>
<th>Healthy</th>
<th>Gingivitis</th>
<th>Mild PD</th>
<th>Moderate PD</th>
<th>Severe PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild PD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderate PD</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Severe PD</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>7</td>
<td>4</td>
<td>12</td>
<td>1</td>
<td>28</td>
</tr>
</tbody>
</table>
At baseline, the log median CD4 counts were similar across the groups ranged from 437 to 469 cells/ml in those subjects who were on ART and 568 cells/ml in those who were not on ART. In the group of 45 participants at 24 months, all ART groups demonstrated statistically significant increases in CD4 count (n = 45, P = 0.007 to P = 0.01). At 24 months, median CD4 counts in the group not on ART were 792 cells/ml, on short-term ART were 655 cells/ml, in the long-term ART-not-suppressed group were 783 cells/ml, and in the long-term ART-suppressed group were 647 cells/ml (Figure 4a). In those individuals on no ART, short-term ART, or on long-term ART who were not suppressed, differences in BGI were not significantly associated with changes in CD4 count at 24 months. However, in those who were in the long-term ART-suppressed group, improved BGI/periodontal status was associated with improved CD4 (P = 0.0436). In the long-term ART-suppressed group, univariate analysis did not reach a statistical significance at 12 months (P = 0.058); however, by 24 months, a statistically significant association was detected between improved BGI and increased CD4 counts (P = 0.01). Of interest, in the group of subjects that were on long-term ART and not suppressed, univariate analysis detected highly statistically significant drops in viral load at both 12 months (P < 0.0002) and 24 months (P = 0.0052) (Figure 4b).

Salivary pro-inflammatory cytokine levels were associated with an increased risk of developing moderate/severe periodontal disease The pro-inflammatory cytokine IL-6 was measured in the oral fluids from HIV-positive individuals (n = 26) (Figure 5). Age-adjusted predicted probabilities (95% CI) of moderate/severe periodontitis were determined. Those individuals with the highest IL-6 levels had a 60% probability of moderate/severe periodontal disease development at baseline (Figure 5a). Importantly, dental intervention resulted in overall decreased salivary IL-6 levels for all diseased groups, with the moderate group, P2, showing a
significant decrease from baseline to 24 months (P = 0.043) as determined by the Wilcoxon signed rank test (Figure 5b). At 12 months, IL-6 levels were assessed across ART groups. Mean IL-6 levels were highest at baseline in the long-term virologically suppressed group and demonstrated a significant decrease from baseline to 12 months (P = 0.031, Wilcoxon signed rank test) (Figure 5c).
Figure 5 Pro-inflammatory Cytokines increase with increasing risk of developing moderate/severe periodontal disease in (a) The likelihood of developing moderate/severe periodontal disease increased with increasing salivary IL-6 in HIV-positive individuals, (b) IL-6 levels stratified by BGI, and (c) changes in IL-6 levels at baseline and 12 months based on ART.
Discussion

Oral microbial load and associated inflammation may influence both local and systemic disease outcomes. In a group of HIV-positive subjects who were virologically suppressed at baseline, dental intervention was associated with decreased periodontitis, increased CD4 counts (P = 0.023) and decreased IL-6 (P = 0.03). While the data presented in this study do not remove the possibility that effective ART was responsible for viral load and CD4 count change in the other ART groups, it does suggest that decreased oral infection and inflammation were associated with improved HIV metrics.

Inflammation is an important driver of both periodontal disease and HIV progression. Our observation of the utility of classification systems that include a measure of inflammation contributes to this literature. Increasing evidence suggests that the chronic periodontal infection is implicated in the generation of a systemic inflammatory response, which represents a potential risk factor for worsening systemic conditions\textsuperscript{87,88}. Use of a periodontal classification system that reflects the biology of disease is an important metric in those with systemic inflammation-associated disorders. This was well illustrated in this study of HIV infected subjects with gingival inflammation.

Of 73 individuals in our population, 63 were on ART and significant inflammation was detected that resolved with dental intervention and aggressive oral hygiene. Distinct profiles of periodontal disease and disease resolution were detected comparing different periodontal classification systems. While AAP provided an important glimpse of historical disease based on attachment levels, inclusion of probing depths in the CDC/AAP classification facilitated biological relevance. BGI included bleeding on probing as a metric, an important indicator of
oral and periodontal inflammation. With BGI, the majority of subjects were classified as severe at baseline. In previous studies, bleeding on probing was twice as high in an antiretroviral-untreated group compared to those on ART\textsuperscript{98}. In our study regardless of ART status, 80\% of subjects had moderate/severe disease at baseline using a classification system that includes current inflammation. Our findings were similar to the findings of John et al in a South African population where HIV stage and ART were not associated with higher levels of periodontal disease in HIV-positive subjects. In the South African group inclusion of a gingival index of inflammation reversed the significant association between antiretroviral therapy, probing depth and clinical attachment loss\textsuperscript{99}. Of all classification systems, BGI, the inflammation-based classification system, demonstrated the most significant shifts in category associated with the dental intervention, moving virtually all individuals of the severe category into a moderate disease group by 12 months. These findings demonstrate the importance of including inflammation as a disease indicator. The periodontal field has now recognized the importance of this indicator, and a recent task force was convened to address the addition of bleeding on probing to the AAP classification to begin 2017\textsuperscript{100}.

In a Nationally representative sample, NHANES 2009-2012, moderate/severe periodontitis was positively associated with non-white race, aging, smoking, male gender, and low income\textsuperscript{86}. In our study of HIV positive individuals, frequency of periodontitis was high across all demographic variables, including gender, age and income. Interestingly, in our study there was not a significant difference with regard to smoking status. HIV may be a more important driver of the oral inflammatory process than the traditional demographic risk factors typically associated with periodontal disease in a nationally representative sample.

Statistically significant decreases in HIV viral load were detected in the long-term ART
group who was not suppressed at baseline. The presence of detectable viral loads, however, may signify ineffective ART. Given that there were individuals with detectable viral load on ART, the improvement in HIV status could have been related to more effective ART use. At baseline, 50% of individuals on long-term ART in the severe disease group were undetectable, and by 24 months, two-thirds of subjects in this group were undetectable. The dental intervention decreased oral microbial load and was associated with decreased low-level HIV viremia. Detectable HIV viral load has been associated with the presence of oral pathogens. A recent Brazilian study determined that detectable HIV VL was associated with elevated levels of known periodontal pathogens, such as P. nigrescens, T. forsythia, and E. corrodens\textsuperscript{101}. There is also the potential for a direct pathogen–pathogen relationship, as others and we have shown that periodontal bacterial end products can increase HIV replication\textsuperscript{102}.

Periodontal disease is associated with circulating microbial products. Bacterial translocation into systemic circulation from the periodontal pocket is a common event, as supported by the detection of bacteremia subsequent to relatively minor periodontal events and procedures. The massive bacterial load of the gut is thought to drive microbial translocation, causing HIV-related systemic immune activation\textsuperscript{93}. Importantly, oral antigens have been shown to facilitate trafficking of activated oral antigen-specific intestinal T-cell responses through CD18\textsuperscript{103}. Hence, periodontal antigens may facilitate intestinal immune activation. We posit that the mouth contributes to microbial translocation in HIV-associated systemic immune activation. While periodontitis does not cause atherosclerotic vascular disease, periodontal interventions do result in reduced systemic inflammation\textsuperscript{104}. Statistically significant improvement in BGI classification was detected in individuals who were virologically suppressed, suggesting improved local oral inflammation and supporting these previous results. Limitations were related
to the study size of this convenience population, particularly the small sample size of the virologically suppressed group. This does limit generalizability. However, this population and its distribution are similar to the demographic distribution of the HIV epidemic in the southeastern US. Despite the limitations, statistically significant changes were detected in the virologically suppressed group. Another limitation was retention rate at 24 months; baseline to 12 months there were 73 subjects followed longitudinally, at 24 months there were only 45 subjects seen at all 5 visits. Importantly, despite these limitations, in every group with detectable HIV VL at baseline, HIV VL was reduced with the intervention.

It has previously been shown that the achievement of undetectable HIV VL was associated with decreased risk of comorbid events and strongly associated with increased CD4 cells\textsuperscript{105}. Low CD4 counts have previously been associated with chronic periodontitis in cross-sectional studies\textsuperscript{106}. In this study, a median sustained increase in CD4 count of over 100 cells/ml was detected with the dental intervention in a group of subjects who were suppressed at baseline. This suggests that over and above ART, dental interventions that diminish the oral microbial reservoir may provide a significant benefit to the immune system.

Periodontal interventions in HIV significantly reduced periodontal inflammation that may be associated with systemic inflammation. Additional studies of systemic immune activation markers and periodontal disease resolution are needed. Here, we describe a simple and relatively inexpensive dental intervention that achieved decreased oral IL-6 and increased CD4 counts in a subset of individuals on effective ART.
CHAPTER 3: CONCLUSION

From its early description in Ancient Egyptian times to the 21st century, our understanding of periodontal disease continues to evolve. Over the past 100 years, the periodontal literature has described the association with bacteria and periodontal disease. Since Van Leeuwenhoek’s description of microscopic organisms, bacteria have been isolated from periodontal pockets, visualized under the light microscope, and cultured. There is no denying the role of bacteria in periodontal disease. However, recent advances in molecular technology have revealed different players involved in this very complex disease, including uncultivable bacteria, viruses and inflammation.

There is evidence that viruses are associated with periodontal disease. Numerous association studies have identified high prevalence of viruses in severe forms of periodontal disease and low levels in health and mild forms of disease. At the molecular level, bacterial by-products, such as SCFAs and LPS, have been shown to lead to HHVs and HIV reactivation in latently infected immune cells. This process may result from microbial translocation and has significant impacts on individuals living with HIV.

Over 20 million individuals are infected with HIV globally, with 1.2 million in the United States. Although many individuals are on HAART therapy, high amounts of systemic inflammation have been reported in the literature. Microbial translocation is particularly
concerning in HIV positive individuals, as bacterial by-products have been shown to lead to reactivation of HIV in latently infected immune cells. The literature mostly describes the gut as the source of microbial translocation with little to no description of the mouth. The oral cavity is a highly contaminated environment; with of over 700 bacterial species and transmucosal teeth surrounded by billions of bacteria, the opportunity for microbial translocation is substantial, especially given the fact that nearly 50% of individuals have some form of periodontal disease. Thus, these bacterial by-products can easily pass through ulcerated gingiva and into the systemic circulation, and trigger immune reactivation of HIV. The contribution of periodontal disease to HIV immune status and vise versa is not well understood and has not been assessed in a longitudinal trial.

There are distinct clinical and epidemiological differences in periodontal disease between HIV positive and HIV negative individuals. Those infected with HIV have a higher prevalence of periodontal disease and the demographic risk factors that are typically associated with an increased odds ratio for developing periodontitis do not show a clear relationship between their presence and increased odds for disease; instead, odds for moderate/severe periodontitis are high, regardless of demographics, indicating that having HIV overrides demographic variables.

Determination of active periodontal disease status is important in HIV patients, as it has been demonstrated that inflammation is an important driver of HIV immune status. Thus classification systems that are more biologically based, such as BGI, should be considered above the more traditional classification systems, such as AAP, which classifies disease based on past history of periodontal destruction. Thus, BGI determines active disease and disease resolution whereas non-biologically based classification systems poorly discern between active and non-active disease.
Reduction of oral microbial translocation and resolution of periodontal disease through treatment leads to significantly reduced inflammation, significantly increased CD4 counts, and significantly decreased VL counts. Thus relatively inexpensive and simple periodontal treatments, including oral hygiene instruction, scaling and root planning, periodontal debridement and maintenances may lead to profound, significant improvements in HIV immune status.
REFERENCES


