The Oral Microbiome and Subclinical Herpesvirus Shedding in HIV Infected Men on ART

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Abstract

Despite undetectable plasma HIV load and CD4 counts reconstitution due to the effective Antiretroviral Therapy (ART) people living with HIV (PWH) are still at a higher risk for developing non-AIDS-associated chronic diseases, which have been linked to systemic immune activation and inflammation. Human microbiota composed of bacteria, viruses and fungi plays an important role in the induction and function of the host immune system and the symbiosis and dysbiosis of fecal and/or oral microbiome have both the local and systemic effects on the immune system. We hypothesize that oral microbiome dysbiosis of PWH leads to systemic immune activation and contributes to non-AIDS-associated chronic diseases. The objective of this study was to evaluate whether the oral microbiome of PWH on ART is associated with levels of systemic immune activation and oral herpesvirus shedding. Thirteen HIV-infected participants on ART and 11 age matched HIV-negative controls participated in this study. Two oral samples with 6-month intervals were collected from each participant. The DNA was isolated from the samples, and the 16S rRNA V4 region was amplified of the DNA by PCR followed by sequencing using Illumina MiSeq platform. The sequence data were analyzed using Qiime2 and statistical analysis was performed by Stata. The results show there were no statistically significant differences in alpha diversity and beta diversity of oral bacteria at family level between PWH on ART and negative controls at both week 0 and week 24 time points,

respectively. There are also no significant correlations between alpha diversity of oral bacteria and plasma inflammatory cytokine levels generated from the previous study. However, when the data were analyzed with herpesvirus shedding, which was obtained from a previous study using the same oral samples, alpha diversity of oral bacteria was significantly decreased in participants with HHV6 shedding compared to those who had no HHV6 shedding regardless of HIV status. In addition, the beta diversity of oral bacteria is also significantly different based on HHV6 shedding status in these oral samples. The same phenomena were not observed based on EBV, CMV or HHV8 shedding status. Our results suggest there may be intricate interplays in the oral microbiome. Future studies with a larger number of participants in each group are needed to determine the role of the oral microbiome in the systemic immune activation observed in PWH on ART. Among PWH on ART, a significant public health concern exists regarding the role of oral microbiome effect on immune activation. There is an imperative need for research to lead to a better treatment strategy concerning non-AIDS chronic diseases in this population.

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Preface

Included in my comprehensive studies in the Infectious Disease Microbiology program, I had the fortunate experience to work with Dr. Yue Chen and Associates examining the role of oral microbiomes in HIV associated pathogenesis. We worked under the hypothesis that the microbiome dysbiosis plays a role in the pathogenesis of non-AIDS associated chronic diseases in HIV infected subjects on ART. I am deeply grateful to Dr. Chen for her guidance and assistance with our study and my interest in this project has motivated me to expand my knowledge of the Human Herpesvirus-6 (HHV6) and its role in the public health community. I deeply appreciate the assistance of Dr. Macatangay and Dr. Martinson for their valuable contributions to my work, especially for their time and service as members of my committee to make my project a success. These three individuals provided invaluable assistance, guidance, and inspiration for my work and interest in this area of a major public health concern. Their time and effort devoted to my study have played a significant role in my success in the Infectious Disease Microbiology program and for that I will always be grateful.

1.0 Introduction

Human microbiome dysbiosis, defined as “a state of community disturbance” is a major cause of microbiome-related systemic diseases.[41] The human oral cavity is the primary gateway to the acquisition of great numbers of bacteria defined as the human oral microbiome. An oral infection caused by a range of microorganisms can lead to system infections such as cardiovascular disease, diabetes mellitus, rheumatoid arthritis, and Alzheimer’s disease.[41] The human oral microbiomes also exert influence on other body functions such as the immune system and physiological functions.[41]

In addition, using 16S rRNA gene based techniques it is estimated that there are between 500-700 bacteria species in the human mouth, and there are no significant differences in microbiome communities based on gender, age, and race.[4]

Human mucosal tissue is the most common route of HIV transmission, but which rarely occurs from oral mucosa.[19] However, compared to HIV negative counterparts, PWH are more likely to have other oral infections including the herpesvirus even when they are receiving antiretroviral therapy (ART).[19] The oral dysbiosis in PWH is hypothesized to impact other parts of the body through immune activation causing systemic inflammation. The oral cavity is continuously exposed to external stimuli, which can introduce the infectious agents to the mouth and the entire body.[19]

The purpose of this thesis is to examine the relationship between the oral microbiome and HIV associated immune activation and herpesvirus shedding status.

1.1 HIV pathogenesis and treatment

HIV infection proceeds in three distinct stages: acute infection, clinical latency, and AIDS.[32] Acute HIV infection begins within two to four weeks post HIV transmission. Individuals may present with flu-like symptoms, but up to half of infected individuals may not present classical symptoms during acute infection. During this phase, many copies of the virus are being produced in the infected individuals. When the virus is replicating, host CD4+ T-cells are slowly being destroyed, which compromises the host's immune system.[32] In the chronic infection stage the virus continues to replicate in blood and lymph nodes of the host.[32] The final stage of HIV infection is the progression to AIDS, which occurs at a median of 8 years from infection if the individual is not on ART. A key component of AIDS is defined by the increased risk of developing opportunistic infections (OI) due to the severely compromised immune system. Individuals can become virally suppressed at any stage of infection by administration of antiretroviral treatment (ART). PWH may not progress to AIDS if viral suppression is achieved through the use of ART.[30]

The current and most effective approach for the treatment of PWH is suppression of viral replication through ART. The majority of PWH receive a combination of antiviral drugs. The goal of this treatment is to provide a potent enough ART to prevent viral replication and drug resistance by reducing plasma viremia to below the limit of detection of commercial assays.[30] The immune function often recovers rates of and AIDS-related clinical diseases sharply decrease. The negative aspect of current ART includes the high cost of medications and care, the need for lifelong therapy, and the emergence of drug resistant HIV if there is incomplete adherence to the medication. There are also concerns of long-term toxic effects of small-molecule drugs. However, although ART is very effective, PWH still have a high prevalence of

non-AIDS chronic diseases, which is believed to be due to immune activation and chronic inflammation.

1.2 HIV pathogenesis and microbiome

Microbiome or small biome is an ecosystem in which all microorganisms that exist in a particular environment constitute a comprehensive interactive system. The microbial communities have been studied using 16S ribosomal RNA (rRNA) gene sequencing technology, which has been widely used to determine the role of the microbiome in the host's metabolism, physiology development, and resulting disease development.[21]

The 16S rRNA gene is present in almost all bacterial genomes. 16S rRNA sequencing was first used in 1985 for bacterial phylogenetic analysis. It has since become the most widely used gene marker in studying bacterial communities. Targeted next-generation sequencing of 16S rRNA is the first molecular method used to study human microbiota. It results in a quantitative description of the bacteria found in biological mixers, and entire biological communities and identifies its constituent members.[52]

The 16S rRNA gene consists of conserved and variable regions. The sequences of the nine hypervariable region (V1-V9) separated by highly conserved regions can provide species-specific signature information useful for identification of bacteria. In targeted next-generation sequencing of 16S rRNA gene method, the hypervariable region is PCR amplified using universal primers targeting conserved regions and sequenced to identify a wide range of existing constituent microorganisms.[52] Since V4 was found to have good domain specificity, high

coverage, and a broad spectrum in bacteria, targeted sequencing of 16S rRNA V4 region is currently widely used in microbiome studies.

Although mechanisms of chronic immune activation in PWH are not fully understood, one mechanism suggested by Brenchley et al contends that disruption of mucosal barriers may permit bacterial translocation into the bloodstream in PWH. PWH are known to have elevated plasma levels of sCD14, sCD163, and IL-6, which have been linked to increased morbidity and mortality of PWH on ART.[10] In humans, the oral cavity has the most abundant microbiomes after those found in the gastrointestinal tract. Oral microbiomes have a direct influence on human health especially through systemic immune activation.[44] Gao et al reported that interactions of well-balanced oral microorganisms help protect the human body from undesirable bacterial and viral invasions. Healthy eating and proper oral hygiene are important to keep oral equilibrium in a positive state.[44] A number of factors such as diet, the use of tobacco, alcohol consumption, poor oral hygiene, and diseases such as diabetes and gingival inflammation can disrupt the microbiome balance. In oral cancer patients, most of whom have a history of smoking, the oral cavity contained significantly higher amounts of streptococci, *Enterococcus,* and *Pseudomonas aeruginosa*. Microbiome changes were also observed in periodontitis, severe dental cavities and gingivitis.[15]

Kistler et al studied the interaction of oral conditions and disease associated pathology in PWH. There were differences in the microbiota of the saliva between the HIV positive and HIV negative groups. Their findings showed that the microbial diversity was lower in those who were HIV+ than those who were HIV-. They also found increased levels of pathogenic bacteria in untreated HIV infected individuals compared to the control group.[18]

1.3 HIV pathogenesis and herpesvirus activation

Human herpesvirus infection and reactivation may cause chronic immune activation in PWH on ART. HHV infections are usually acquired by close contact with oral secretions during early childhood and by sexual activity or blood transfusion later in life. The prevalence of human herpesvirus infections is very high in the general population. Most people with normal immune systems carry these viruses asymptomatically.[1] If an individual is immunologically compromised, such as with untreated HIV infection, then these common viruses can become a source of disease. In PWH, there is a greater increase of disease of both infections and cancers caused by oncoviruses EBV, HHV-8 and HPV [44]. Verma et al studied the role of viral infections that coexist with oral cancers and other systemic diseases, and found that immunosuppression caused by HIV infection results in an immune response complicated by otherwise tolerable viruses.

Recently, Agudelo-Hernandez et al reported that herpesvirus shedding rates are higher among HIV+ infected men who have sex with men (MSM) despite viral suppression and CD4+ T cell reconstitution.[2] The authors showed that PWH on ART in the study had higher rates of EBV and CMV shedding as compared to the controls. The level of sCD14 cytokine was significantly associated with increased levels of shedding status for EBV. CMV shedding rates were not associated with any cytokine biomarkers, but were inversely related to T-cell activation. Cytokines IP10 and sCD163 negatively correlated with the HHV6 shedding rates. This suggested that reactivation of HHV6 from latency may be driven by lower levels of inflammation.

1.3.1 Dynamic changes of oral microbiome in HIV infection

The microbiome can be assessed by the alpha and beta diversity measurements.[40] Alpha diversity is defined as the variety and abundance of organisms in a single community, beta diversity is defined as differences in abundance between organisms in different community.[40] Decreased microbial diversity and richness in the GI tract have been reported to be correlated with increased risk of cardiovascular diseases, certain cancers, and inflammatory diseases.[47] The oral cavity harbors a large amount of bacteria that are essential for the development of an innate as well as adaptive immune system. Bacterial diversities within the oral cavity can be altered at any point with the change of HIV serostatus, and with the chance of acquiring an opportunistic infection. PWH have been reported to have a lower alpha diversity of bacteria living within their oral cavities.[31]

Bacterial development in the oral cavity leads to multiple periodontal diseases, but only a few harbor the ability to develop and sustain infection. When herpesvirus shedding occurs, a bacterial-viral interaction leads to progression of the infection, which not only results in periodontitis, but sustains the infection itself.[34] The lesions that reside in the mouth because of periodontal infections manifest an elevated level of bacteria. When the herpesvirus is active, the systemic immune system, especially the mucosal immunity that resides in the oral cavity, can cause localized immune defenses to decrease.[34] Overall, this study suggested that the interactions between the oral cavity with both periodontal infection and herpesvirus infection coincide with the bacterial diversity in the human mouth.

Gianella et. al reported in ART-naïve PWH, there is a positive correlation between plasma HIV DNA levels and herpesvirus shedding in blood**.** Furthermore, in the PWH on ART, over 60% of the cohort had some type of HSV virus. Many of them had higher shedding rates

despite being virally suppressed on ART.[11,12] Their study demonstrated that herpesvirus shedding was higher among PWH with or without ART compared to seronegative controls.

Lusso et al developed research methods delving into the correlation between Simian immunodeficiency Virus (SIV) with HHV in macaques. The primates infected with SIV showed stable CD4+ and CD8+ T cell counts when not inoculated with the HHV6 strain. Once primates were superinfected with the HHV6, rapid CD4+ T cell depletion occurred.[22] The coinfection results of HHV6 with SIV in primates suggest that similar coinfection of HIV and HHV may accelerate the progression to AIDS.[22] After further research, the authors concluded that the HHV6 coinfection directly affects the basic pathogenic mechanism of immunodeficiency viruses.

2.0 Hypothesis and Specific Aims

2.1 Hypothesis

Oral microbiome dysbiosis among PWH on ART is associated with higher levels of immune activation and systemic inflammation.

2.2 Specific Objective

The specific objectives of this study are:

1. Measurement and analysis of oral microbiome in PWH on ART and age-matched HIV negative controls.
2. Analysis of the oral microbiome profile with oral herpesvirus shedding status and soluble markers of inflammation.

3.0 Methods

3.1 Study subjects and sample collection

Archived frozen samples from 24 participants from the Multicenter-AIDS Cohort study (MACS) were used in this study.[2] The MACS is a longitudinal prospective study of MSM. Oral wash samples along with blood, semen, urine and stool were collected from 13 HIV (+) participants who have been virally suppressed on ART for a median of 7 years and 11 HIV uninfected controls at Weeks 0, 4, 8, and 24 of the study. Oral wash samples collected at week 0 and week 24 were used in this study.

3.2 DNA isolation from oral wash samples

DNA was extracted from oral wash samples collected from week 0 and week 24 using the Power Soil Extraction kit (Mo Bio, USA) based on the manufacturer's instruction with minor modification. Briefly, 500ul of oral wash sample was dispensed into a tube containing sterile beads and vigorously vortexed for 10 minutes. Following multiple washing steps, the DNA was eluted from the beads with 100ul of elution buffer and saved in -20C. To control the quality of the DNA isolation procedure, positive and negative control tubes were included for each DNA isolations run.

3.3 Polymerase chain reaction of oral wash samples

After the completion of the DNA isolation process, PCR was performed on the isolated DNA to amplify the hypervariable region (V4) of the bacterial 16S rRNA gene with the following primers: 16S Amplicon PCR forward Primer=5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG :16S Amplicon PCR Barcode tagged Reverse Primer= 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

The PCR reaction with total volume of 25ul containing 4ul of extracted DNA was run at the following cycling conditions by the Caporaso et. al method. Thermocycle conditions were 98C for 30s, followed by 25 cycles of 98C for 10s, 57C for 30s, 75C for 30s. After the cycling samples were run 72C for 2min, and lastly PCR product was held at 4 degrees. At the completion of cycling parameters, 5ul of PCR products were run on a 2% agarose gel to visualize a band formation at the 400 base pair mark ensuring that PCR product was amplified for each oral wash sample.

3.4 Clean-up of PCR products

Following the PCR amplification of bacterial 16S rRNA V4 region, the PCR products were cleaned from primers, enzymes and the buffers and selected for 200-600bp using SPRIselect for size selection kit (Beckman Coulter) following manufacturer's protocols. The cleaned and size-selected PCR products were stored at -20C for Quantitative analysis by QuBit Fluorometer.

3.5 Quantitation, pooling, and sequencing of PCR products

A Qubit Fluorometer can be used for the quantification of DNA, RNA, or protein of a sample. After the completion of a PCR clean-up and size-selection, the DNA contents of the PCR products were quantitated by a qubit fluorometer using a Broad Range Kit from Life Technologies following manufacturer's instructions. The ideal DNA concentration for subsequent pooling procedure is between 2-15 ng/ul. If the DNA concentration of the PCR products were lower than 2ng/ul, a PCR would need to be redone on that sample to generate more PCR product. The DNA concentration of oral wash samples 5, 13, 29, and 39 was below 2 ng/ul resulting in a required repetition of PCR.

The pooling of the cleaned PCR products was performed by combining 40 ng of DNA from each sample into one tube. After final clean-up of the pooled DNA using the Purelink PCR purification Kit (Life Technologies), the PCR products with PhiX controls were sequenced using MiSeq.

3.6 Bioinformatics analysis of oral wash Sample

The sequence reads from the respective PCR products were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2), the bioinformatics software package for taxonomic analysis. Trimming of the sequences was performed in QIIME2 to select the best quality portion of each sequence for subsequent analysis. Next, the QIIME2 software, gg-13-8-99-515-806-nb-classifier was used as a reference base for an operational taxonomic unit (OTUs) assignment for the sequence reads. This software developed a baseline taxonomic assignment for oral bacterial

composition. The alpha diversity of the bacteria was determined by the Shannon Diversity Index method and beta diversity was determined by the use of the Weighted UniFrac method implemented by QIIME2.

3.7 Measurement of herpesvirus shedding rates

Using the same collected oral samples Macatangay et al and colleagues measured herpesvirus shedding in a previous study. Briefly, DNA extraction was performed for all samples with EasyMag (bioMerieux, Durham, North Carolina, USA). Following extraction, Real-Time Taqman quantitated PCR was performed for quantification of herpesvirus in the samples. Master Mix comprised of 5uL sample DNA or control, 10uL of Taqman 2X Gene Expression Master Mix (Applied Biosystems, Foster City, California, USA), 1,2 µmol/L forward and reverse primers, and 0.3 µmol/L of probe. All samples cycle conditions were: 50ºC for 2 min, 95ºC for 10 min, followed by 45 cycles of PCR amplification, 95ºC for 15s and 60ºC for 1 min.

3.8 Measurement of soluble inflammation markers

In previous study Macatangay et al used commercially available ELISA kits to measure soluble inflammation markers sCD14, sCD163, interferon γ-induced protein 10 (IP-10), interleukin-6 (IL-6), and C-reactive protein (CRP) following manufacturer's instructions. Inflammation markers were analyzed by using ELX808 ELISA Gen5 software v2.06 (Biotek, Winooski, Vermont, USA).

3.9 Statistical analysis of oral wash samples

STATA statistical analysis packages were used for the analysis of the data generated. In QIIME2, the Kruskal Wallis Pairwise test was used to examine the difference of alpha diversity of oral microbiome based HIV status and/or HHV shedding status. The pairwise Permanova test in QIIME2 was used to obtain beta diversity p-values. All p-values were assessed at a confidence level of 95% or a p-value ≤ 0.05. P-values at or below 0.05 were considered to be statistically significant. A Bonferroni correction for multiple independent observations was applied for the shedding status tests.

4.0 Results

4.1 Study Participants

Table 1 provides specific information regarding the participants of this study. There were 24 study participants, 13 HIV (+) and 11 HIV (-) with a median age of 41(range=27-50) The HIV (+) participants have been infected for a median of 10 years (range=3-19) and had been virally suppressed on ART for a median of 7 years (range=1-7). Participants have a median CD4+ T cell count of 864 cells/mL (range=533-1355)

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Table 1. Clinical characteristics of study participants

Characteristics	HIV(+)	HIV(-)
N	13	11
Median age in years(range)	41 (27–50)	41 (26–50)
Race		
Caucasian	10	10
African-American	3	1
Median years HIV-infected (range)	10 (3–19)	
Median years ART suppressed (range)	7 (1–10)	
Median CD4 T-cell count (range)	864 cells/ml (533–1355)	

4.2 Oral microbiome sequence and analysis based on results between HIV Status of study participants

The completion of sequencing runs generates the sequence for each sample. Figure 1 shows the results of sequences reads of the 16S rRNA V4 region of all oral wash samples in this study. Along the X-axis, the frequency reads per sample is compared to the Y-axis alignment demonstrating the number of samples at a given frequency.

The sequence read frequencies ranged from a low of 34,201 counts/sample to a high of 85,379 counts/sample, with an average of 49,413 counts/sample (Table 2). These results indicate that even the lowest counts (34,291 counts/sample), using the standard of 30,000 counts/sample as an acceptable norm (Caporaso et al), provided sufficient sequence counts for the subsequent sequence analysis.

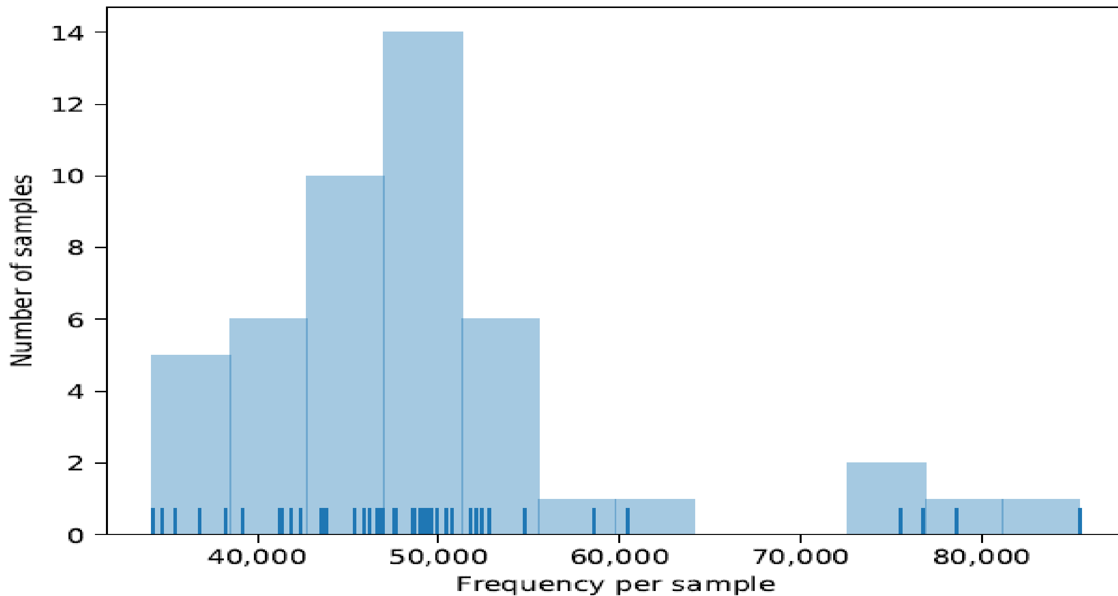

Figure 1 Quality score and parameters of sequences Generated

Table 2 Summary table of microbiome sequencing run

Summary Table	
Number of Samples	47
Minimum frequency	34201
Maximum frequency	85379
Mean frequency	49413

Taxonomy plots generated from sequence analysis using QIIME provide a general overview of bacterial species richness in each oral wash sample (Figure 2). The samples were categorized by HIV status and compared the relative frequency in percentages (%) of the amount of each bacterium present at the family level in each analyzed sample. It is evident that there is a wide variety of bacterial content across the samples. The top four phyla were Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria. There was no apparent difference in the ranges of bacteria across samples between the two study groups.

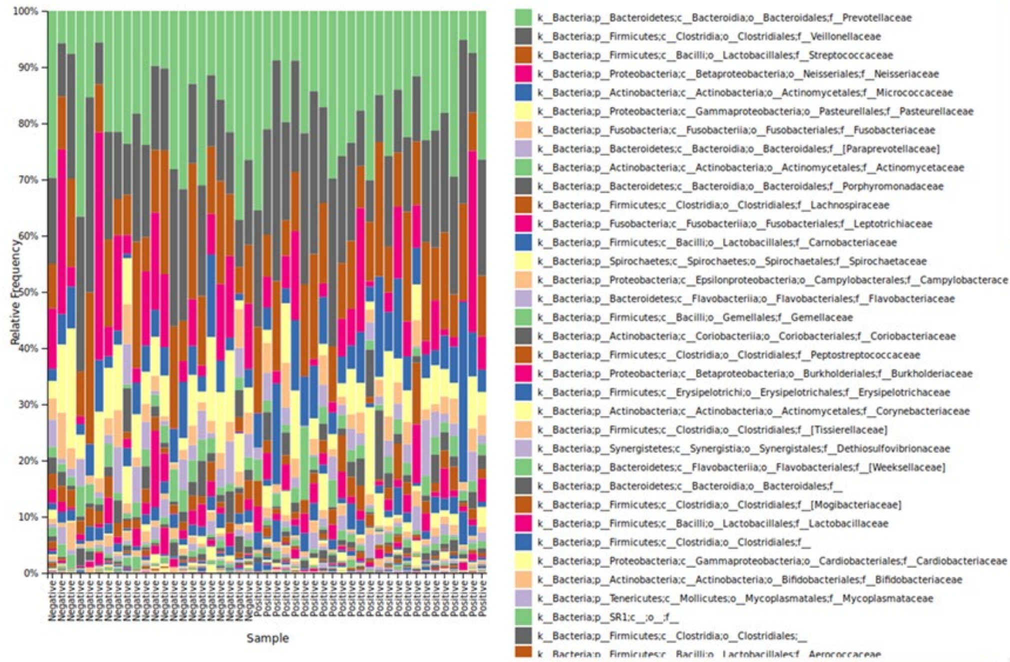

Figure 2 Taxonomy plot at the family level

Sequence analysis also generates alpha and beta diversity based on varieties of bacterial species contained in the samples. Alpha diversity is defined as the bacterial species richness in a given sample. Beta diversity is defined as the similarity or dissimilarity between multiple samples measured by Weighted UniFrac Significance.

Using STATA software, box plots were generated based on three variables, the alpha diversity of each sample generated by Shannon Diversity Index method, week of sample collection, and HIV status. Figure 3A demonstrates a comparison of the alpha diversity at the beginning of the study, week 0and that at week 24. There were no significant differences in the alpha diversity between the two time points (week 0 and 24; p=0.75) suggesting no changes in the oral bacterial diversity within the 24 week period. Similarly, no differences in the alpha diversity were observed between HIV infected participants and uninfected controls (p=0.18)

In figure 3C the levels of alpha diversity were further analyzed based on HIV status, and the sample time points of week 0 and week 24. There were no statistical differences of alpha diversity between HIV+ and HIV- groups at both time points. Between the same time points although there appears to be higher level of alpha diversity in the HIV + group, this did not reach statistical significance.

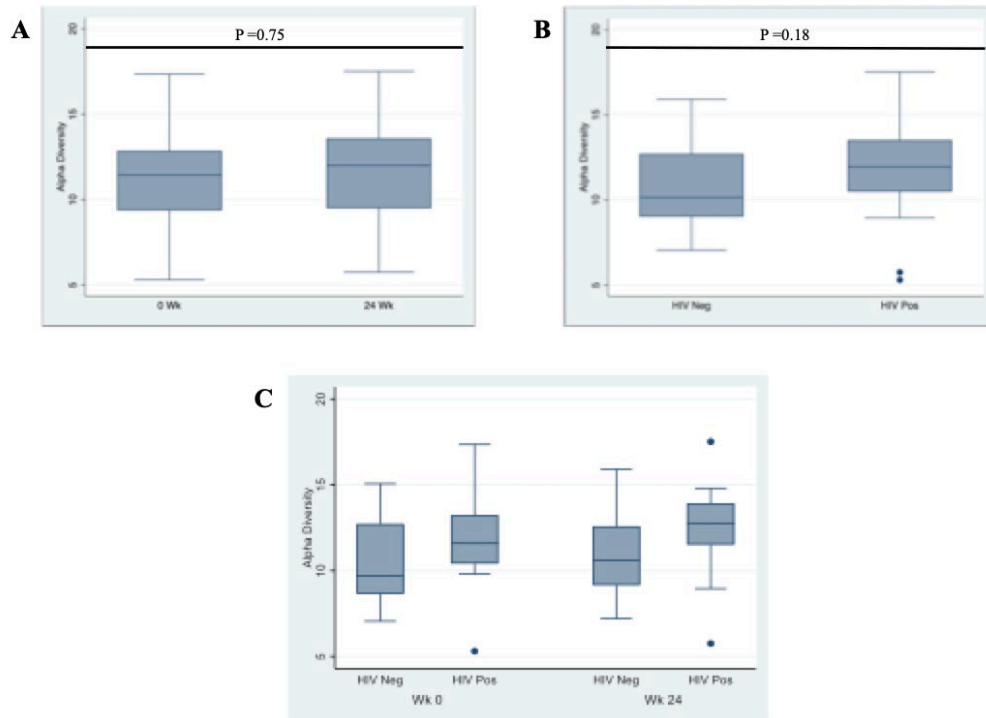

Figure 3 Comparison of Alpha diversity in HIV-infected and HIV-uninfected groups

Principal Component Analysis is used to reduce large amounts of data into a three-dimensional space. Data points similar to each other will cluster closer together. Bray Curtis plots based on beta diversity by Weighted UniFrac method in QIIME2 were generated to define the difference in microbial composition between samples (Figure 4). Axis 1, 2, and 3 showed the percent of variance between the samples that is explained by the first three principal components respectively. In figure 4A beta diversity was generated based on HIV status. Figure 4B shows the beta diversity between samples based on the week of sample collection. Lastly, Figure 4C shows beta diversity based both on HIV statuses and sample collection time points. However, there are no significant differences between the beta diversities based on a Pairwise Permanova test with all p-values >0.05 from Figure 4.

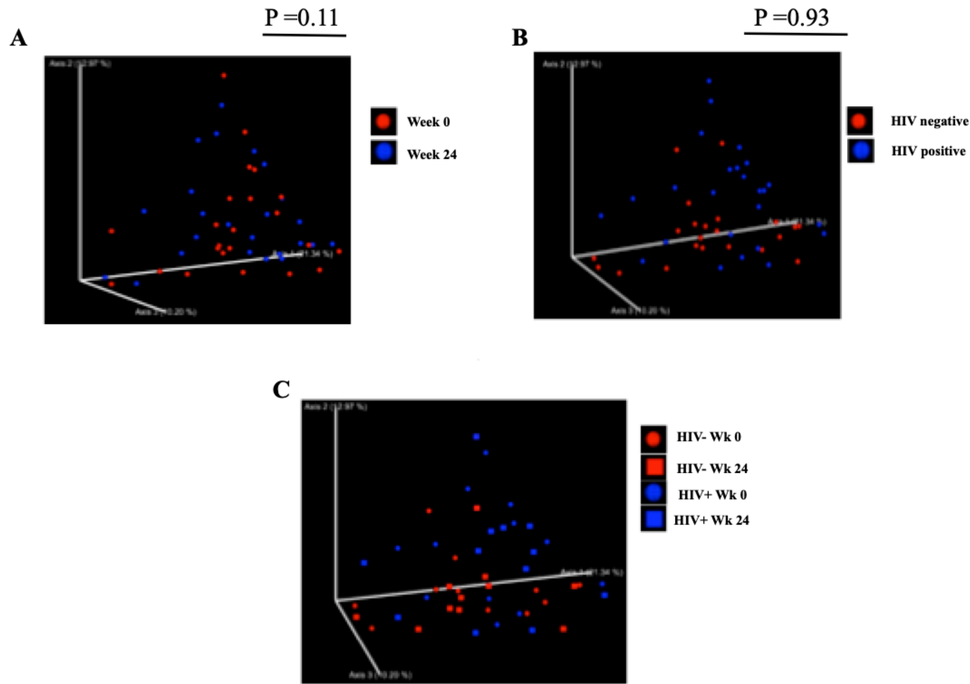

Figure 4 Bray-Curtis PCA plot of oral wash samples

4.3 Analysis of oral bacterial alpha diversity with HHV shedding status and cytokine levels

In a previous study, multiple human herpesviruses such as HHV6, HHV8, EBV, and CMV were measured in the oral wash samples which were regarded as shedding negative if no virus was detected or shedding positive if the virus was detected. In figure 5A, B, C and D, levels of alpha diversity were compared based on HHV6, HHV8, EBV or CMV shedding statuses at week 0 and week 24.

The alpha diversity of each sample was further evaluated based on herpesvirus shedding status to assess the effects of a herpesvirus shedding on oral microbiome (Figure 5). The results show there were significantly lower alpha diversities in HHV6 shedding samples compared to no

HHV6 shedding samples with a p value of 0.009 regardless of HIV infection status. To confirm, Bonferroni Correction was applied generating a p=0.0125 resulting statistical significance of HHV6 shedding status. Similarly, alpha diversities were also analyzed based on the shedding statuses of HHV8, EBV and CMV. There are no statistical differences of alpha diversities based on HHV8 shedding status (p=0.29), EBV shedding status (p=0.57), and CMV shedding status (p=0.49) at the 95% CI.

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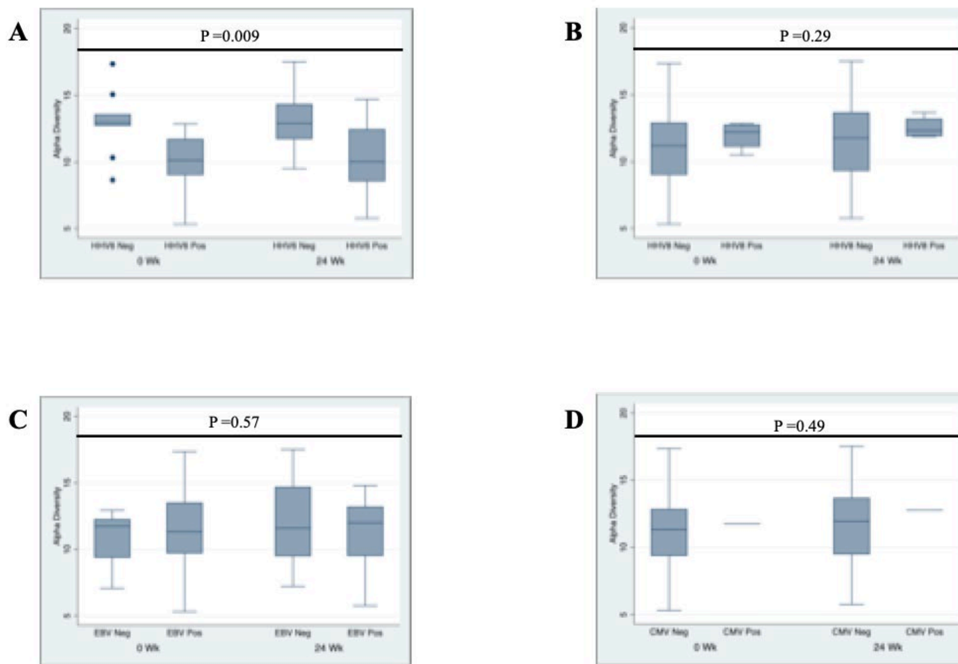

Figure 5 Alpha diversity compared to HHV status with week of sample collection

Beta diversity of oral bacteria was also analyzed based on HHV shedding status (Figure 6). In figure 6A beta diversity was analyzed based on HHV6 status. There is significant difference in beta diversity between HHV6 shedding samples and no HHV6 shedding samples with p=0.008. Bonferroni Correction was applied generating a p=0.0125 confirming statistical significance of HHV6 shedding status. Figure 6B, 6C and 6D demonstrate the beta diversity

based on sample's HHV8, EBV or CMV shedding status. There are no statistical differences in beta diversity based on HHV8, CMV, or EBV shedding statuses.

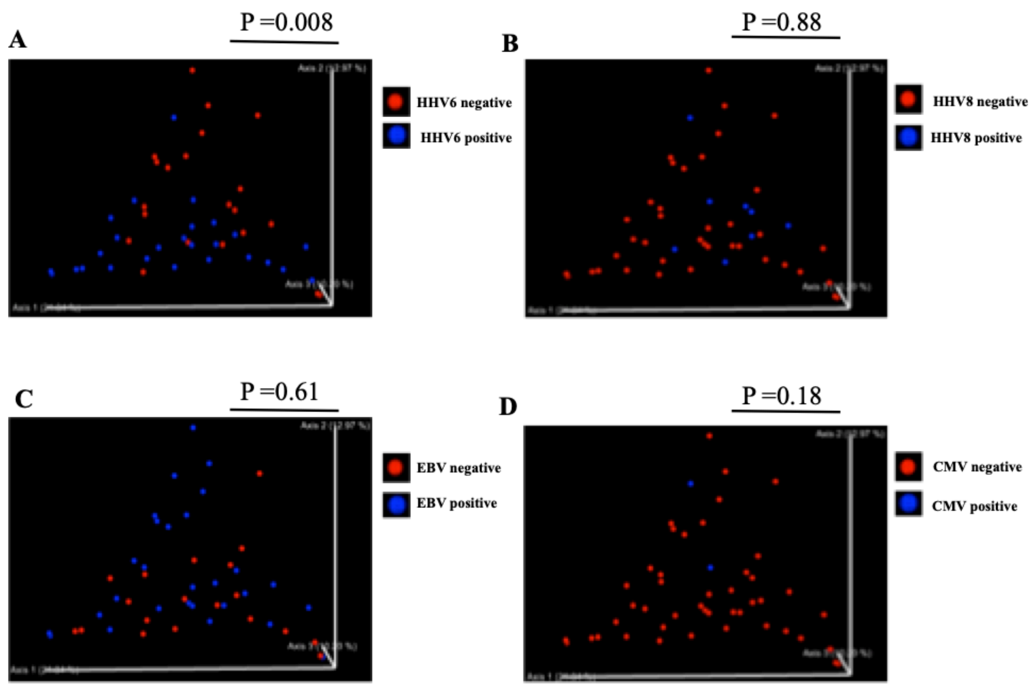

Figure 6 Bray-Curtis PCA plot comparisons between HHV statuses

From the previous study with the same study participants, five soluble markers: IP10, IL-6, sCD14, sCD163, and CRP of inflammation with were measured in plasma to determine the inflammatory cytokine levels at each time point.[2] Multivariate correlation analysis of alpha diversity of oral bacteria and the cytokine levels were performed and no significant correlations were observed between bacterial diversity and plasma levels of the different soluble markers.

5.0 Discussion

The goal of this study was to determine whether oral microbiome dysbiosis among PWH on ART is associated with higher levels of immune activation and systemic inflammation. Persistent inflammation and chronic immune activation result from HIV (+) infection. Metabolic changes in PWH associated with impairment of the immune system resulting in cytokine network changes results in inflammation in both ART treated and untreated individuals.[46] Altered immune system status could lead to non-AIDS diseases in PWH according to Zicari et al. Those non-AIDS diseases include cancers and HIV-associated lipodystrophy syndrome (HALS) characterized by redistributed body fat and adipose tissue damage. The presence of this condition makes these individuals have an elevated cardiovascular risk.

As all adults age, there are documented declines in physical function and the onset of frailty. These are closely associated with inflammation and immune system dysfunction. Among aging PWH there are earlier declines in physical function and developing frailty. Erlandson et al describes three levels of decline; normal aging declines, moderate declines among PWH on ART, and the most severe among PWH not receiving ART. The status of immune activation is best controlled with ART among those who have high CD4+ T-cell counts. Hileman et al proposed ART is effective in reducing persistent inflammation and immune activation but not to the level found in HIV (–) people.

There are different microbial communities in different human body parts due to vastly different conditions on our skin and in our mouths, noses, and guts. The oral microbiome

detected from our study participants was mainly composed of five major phyla (Figure 2): Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria, which are consistent with the findings of numerous previous studies.[10,29,35]

HIV infections are known to influence alterations in both the gut and oral microbiome due to its systemic effects on mucosal surfaces.[29] Also McHardy et al showed that PWH have an altered bacterial richness in both gut and oral mucosa due to the infection. The McHardy et al study also concluded that participants who were virally suppressed on ART treatment had similar bacterial species richness compared to uninfected individuals. As a result, the use of ART may be considered an effective mechanism for maintaining a healthy gut or oral microbiome composition. We measured oral microbiome through the 24-week-study period in both PWH on ART and control individuals. The taxonomy plot (Figure 2) showing the different bacteria at family level displays the similar microbiome profile in both populations at both sample collection dates, which indicates that overall trend of bacterial diversity and richness was similar between PWH on ART and uninfected controls and was fairly stable in all participants over the 24-week-study period.

There are two parameters frequently used in microbiome analysis: alpha diversity and beta diversity of the bacterial species composition. Both the alpha diversity and beta diversity can be largely impacted by the disturbance of the microbiome due to any factors limiting the range of optimization for the community.[37] Alpha diversity is defined as the variety and abundance of organisms in a community or sample; Beta diversity is defined as measuring the similarity or dissimilarity between multiple communities or samples.[45] Our results showed that there is an increase of alpha diversity in PWH on ART group compared to uninfected controls at both week 0 and week 24 time points. However, this increase was not statistically significant.

Currently, it is not clear whether this difference is due to the sample variation or limited sample size in this study. Future study with larger sample size is needed to confirm this difference.

All herpesviruses lead to life-long latent infection with periodical reactivation depending on various local and systemic conditions including HIV infection. Oral herpesvirus infection and reactivation are mostly asymptomatic, which however could disrupt oral microbiome environment and lead to microbiome dysbiosis. Our study showed that alpha diversity levels significantly decreased in participants who were orally shedding HHV 6 compared to the subjects who were not orally shedding HHV6 at both at week 0 and week 24 time points. Furthermore, we show that there is significant difference of beta diversity in the participants orally shedding of HHV6 compared to the participants who were not orally shedding HHV6. There are no significant differences in alpha and beta diversities of oral microbiome when analyzed based on other herpesvirus (HHV8, CMV, and EBV) oral-shedding statuses. Herpesvirus also potentially affects the progression of Alzheimer's disease (AD). The most common cause of dementia is Alzheimer's and evolving research has indicated most studies have shown the association of HHV for AD.[7] Herpesviruses have the ability to become latent in the host and later infect neurons. Studies show HHV6 had a 23% positivity in blood samples from Alzheimer's disease infected individuals. 17% of AD brains were HHV6 positive in the Carbone et al study. This indicates that continued research should be conducted as findings suggest that HHV6 poses a significant risk factor for cognitive dysfunction and may lead to AD in the elderly. Factors to study include viral latency to help clarify the role of HHV6 pathogen in AD.

In a previous study, plasma cytokine markers IL-6, CRP, sCD4, sCD163, and IP-10 were measured in the same study participants at the same study visits.[2] Therefore, we examined the relationship of levels of soluble markers of inflammation to levels of alpha diversity to

investigate the role of oral microbiome in systemic immune activation. Interestingly, there were no significant correlations observed among the soluble markers and alpha diversity. These soluble markers have been associated with persistent immune activation among treated PWH.[18] Previous studies indicate a possible association of continued immune activation as the result of replication of HIV in latent reservoirs. The Gianella et al study found that more than 75% of PWH on ART had at least one actively replicating HIV in their mucosal tissue. This study aligning with our results suggests that HIV replication may be a potential cause of chronic immune activation levels.[16]

There are multiple limitations that exist in this study that could be affecting the results of the project. First, data was obtained from a small study population (N=23) so results may not be generalizable to all PWH on ART. Similarly, the study only includes MSM. Results from women infected with HIV on ART should also be assessed to see whether findings would be consistent with different study populations. Third, we did not monitor the dietary intake and oral hygiene of participants. Previous studies concluded that eating, drinking, and smoking constitute varying factors affecting the oral microbiome of any individual whether the person is HIV infected or HIV uninfected.[39]

Our conclusions did not support our original hypothesis that the oral microbiome dysbiosis of PWH on ART is associated with immune activation and inflammation. Further research is indicated to clarify the discrepancy between the findings of our study and those of previous researchers.

6.0 Public Health Significance

Advances in ART have increased the overall life expectancy for PWH. Even with viral suppression on ART, PWH still have persistent immune activation and elevated levels of inflammation compared to HIV-uninfected individuals. The incidence of HIV and related diseases continues to be significant on an international basis. According to WHO, there are presently nearly 37 million people throughout the world living with HIV. A major aspect of this is the huge demand for specific treatment and a simultaneously critical need for prevention and continuing research.

Studying the microbiomes in the human body, and how shedding effects may cause systemic complications, research is vital considering the magnitude of the public health concern of HIV infections. Immune activation that results from associated microbiome dysbiosis presents a significant effect resulting from the co-infection and comorbidity of the two conditions. Additionally, there is a significant economic impact of this widespread disorder and the systemic diseases that are associated with HIV. Studies of microbiomes have raised the level of alertness of the importance and impact that they have on the health of infected humans. The role of microbiomes in the co-infection process of herpes shedding and HIV indicates the strong need for research, prevention, and effective therapeutic interventions to be developed and implemented.

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