2023

Histone Acetylation in Periodontal Diseases

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

By

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Acknowledgements

I would like to thank my committee members, Dr. Zhao Lin, Dr. Thomas Waldrop, Dr. Ping Xu, and Dr. Caroline Carrico for generously giving their invaluable time and support to my project. The support of such brilliant and esteemed individuals in their fields was indispensable for maintaining confidence in my work and guiding me throughout my residency.

I would like to extend my gratitude to Dr. Lin specifically for the innumerable hours he has dedicated to maintaining an active role in my project, his support went beyond mentorship.

I would like to thank Dr. Lin's lab for their priceless effort throughout this project, particularly Dr. Sheikh Alam, David Pellei, and Joud Al Kallas. I would like to thank Dr. Liang Bao in Dr. Ping Xu's lab for his tremendous work in the microbiome study.

I would also like to thank my family and in-laws who have supported and encouraged me throughout this journey.

Lastly, I wish to express my gratitude and appreciation to the pillar of support and strength in my life, my wife, Dr. Solaleh Shahmirzadi, her support and continuous encouragement over the many years of study was a precious gift.

Table of Contents

List of Tables

List of Figures

Abstract

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Virginia Commonwealth University, 2023

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DEPARTMENT OF PERIODONTICS

Background: Emerging data has indicated the significant role of epigenetic control in inflammation. Acetylation of lysine residues on histones is a major epigenetic modification of chromatin, which is required for cytokine gene expression and regulated in inflammatory diseases. It has been shown that periodontal bacteria induce histone acetylation in oral epithelial cells and experimental periodontitis in rodents. In this study, we investigated the acetylation level of histone 3 (H3) in gingival tissue from healthy and periodontitis patients. We hypothesize that cytokine over-production in periodontitis is associated with the hyper acetylation of histones.

Methods: Periodontitis and healthy subjects who need periodontal surgeries (pocket reduction for periodontitis patients or crown lengthening for healthy individuals) were enrolled under IRB approval. Gingival tissues from diseased and/or healthy sites were harvested during surgeries. Immunohistochemistry (IHC) staining was used to evaluate the expression of acetylated H3(K4+K9+K14+K18+K23+K27). The inflammatory status of tissues were assessed by immune

cell infiltration level under Hematoxylin and eosin (H&E) staining. Subgingival plaques were collected and 16s rRNA sequencing was used to profile the microbiome.

Results: 39 subjects were recruited for this study. 24 patients were included in the diseased group and 15 in the healthy group. Through IHC, H3 acetylation was detected in the nucleus of gingival epithelial cells, fibroblasts, endothelial cells and infiltration immune cells. In the connective tissues, a higher percentage score was seen in periodontitis samples although it was not statistically significant (P=0.0521). There was no difference in the intensity score and total IHC score. Analyses based on tissue inflammatory status showed that the percentage score of acetylated H3 in inflamed tissues was dramatically higher than that in non-inflamed tissues, as well as well as the total IHC score. From 16s rRNA sequencing, 9 species differently presented between periodontitis and healthy pockets.

Conclusion: Within the limitations of this study, a higher level of histone 3 acetylation was present in the inflamed gingival tissues compared to non-inflamed tissues. Our study suggested that histone acetylation may be a potential pharmacological target for treating periodontitis.

Introduction

Periodontitis is one of the most prevalent inflammatory diseases. It can affect the alveolar bone, gingiva, periodontal ligament, and teeth. Periodontal diseases are a major burden for our society, and it is the sixth most frequent health issue in the world.[1,2] The Centers for Disease Control and Prevention (CDC) survey states that 50% of American adults over the age of 30 have periodontitis. [3]

Periodontitis is a multifactorial disease. It is characterized by inflammation and destruction of periodontium apparatus. [4] The disease is initiated by a dysbiotic microbial community in a susceptible host [3]; however, the damage to the tooth-supporting complex is ultimately the result of the host's immunological reaction to bacterial invasions. [5,6]

The goal of current periodontal therapy is to reduce the bacterial load in periodontal pockets through mechanical debridement with or without flap surgeries. [7] However, they are not always effective, and the recurrence of hyper-inflammatory status in periodontal tissues is frequently observed. [8] Despite the fact that periodontitis has a microbial etiology, a number of factors, including systemic diseases, environmental factors, genetic and epigenetic factors, can affect how the condition develops. [4] The susceptibility to periodontitis is largely determined by host response and the severity of inflammation predicts disease progression. Therefore, understanding the driving force of the uncontrolled, overly aggressive inflammation is important for future therapy.

Emerging data has indicated the significant role of epigenetic control in inflammation. The term "epigenetics" refers to a change in gene expression that is not encoded in the DNA sequence. It also refers to chemical alterations of the DNA and the proteins that are related to it (known as histones), which result in the remodeling of chromatin and the activation or inactivation of the desired gene. [9] Acetylation of lysine residues on histones is a major epigenetic modification of chromatin, which opens the chromatin for gene transcription. The status of histone acetylation is usually “translated” by bromodomain and extraterminal domain (BET) proteins, which include BRD2, BRD3, BRD4 and BRDT. BET proteins “read” the status of chromatin by binding to the acetylated lysine on histones (H-Ac), and therefore facilitate the recruitment and activation of transcription factors. [10,11]

Histone acetylation are required for cytokine gene expression and are regulated in inflammatory diseases. It has been shown that periodontal keystone pathogen Porphyromonas gingivalis induces histone acetylation, H3 in specific, in oral epithelial cells, which may be associated with its metabolic product butyrate. [12] In a ligature induced experimental periodontitis model in rodents, H3K9 is hyper acetylated in the gingival epithelial tissue. [13] However, the histone acetylation status in human gingival tissue from periodontitis patients is still largely unknown. In this study we hypothesize that the cytokine over-production in periodontitis is associated with the hyper acetylation of histones. Specifically, we investigated the acetylation level of H3 in gingival tissues from healthy and periodontitis patients. Because epigenomic modification represents a significant interface by which the microbiota dynamically interact with the host genome, we also hypothesize that tissue epigenetic status is associated with specific diversity of microbiome population. To investigate such potential correlation, subgingival plaques were collected and analyzed by 16S rRNA sequencing.

Materials and Methods

Study Design and Participants

The protocol for the study was approved by the Virginia Commonwealth University Institutional Review Board (MS3_HM20013515). The study population included periodontitis patients and healthy subjects who need periodontal surgeries (pocket reduction for periodontitis or crown lengthening for healthy individuals) in the Graduate Periodontics clinic.

Screening Process

Patients were considered for inclusion in the study according to the following criteria: For the healthy group, subjects had no probing depth (PD) >3mm and no clinical attachment level (CAL) >3mm, and had less than 20% of the sites with bleeding on probing (BOP). For the periodontitis group, subjects had at least 6 teeth with PD ≥6mm. Measurements were performed at 6 sites per tooth including mesiobuccal, mid-buccal, distobuccal, mesiolingual, midlingual, distolingual. The exclusion criteria were: under the age of 18, less than 20 teeth, having removable dental appliances or having orthodontic treatment, cancers, severe asthma, oral candidiasis, systemic conditions that required long-term use of anti-inflammatory drugs or immunosuppressants, scaling and root planning in the last 3 months, antibiotics treatment in the last 3 months, pregnancy and breast feeding. (Table 1).

Table 1. Inclusion and exclusion criteria.

Inclusion	Exclusion

• Age range: at least 18 years old
• Patients must be able and willing to follow study procedures and instructions.
• Patients must have read, understood and signed an informed consent form.
• **Group 1: patients stage III or IV (moderate to severe) periodontitis. He/She must have all of the following:**
  ○ More than 5 teeth with probing depth >=6mm, at least 2 teeth in the quadrant that will receive surgery.
  ○ Attachment loss >=3mm at those teeth.
  ○ Who is scheduled for periodontal flap surgeries such pocket reduction surgery, open flap debridement, tooth extraction, alveolar plastic, etc.
• **Group 2: periodontally healthy patients.**
  ○ No probing depth more than 4mm.
  ○ No more than 20% of teeth with bleeding on probing.
  ○ Who is scheduled for periodontal surgeries such as crown lengthening surgery and tooth extraction.
	• Individuals under 18 (minors)
	• Individuals who have had antibacterial antibiotic therapy in the last 3 months.
	• Individuals with < 20 teeth
	• Individuals with active pulp/periapical infection that needs immediate dental treatment.
	• Individuals who are pregnant (by self-report) or lactating
	• Individuals with prior radiation treatment and chemotherapy for malignancy
	• Individuals who are incarcerated or otherwise unable to provide consent freely.
	• Individuals with limited English proficiency.
	• Individuals with active transmissible infections such as HBV, HCV, HIV and Covid-19
	• Individuals with cancers or systemic conditions that required long-term use of anti-inflammatory drugs or immunosuppressants.

Sample collection

Gingival tissue and subgingival plaque samples were collected as shown in Figure 1. They were harvested from “diseased” and “healthy” subjects according to the following criteria: “diseased” sites showed BOP, an interproximal PD of ≥6mm, and a concomitant CAL≥3mm. “Healthy” sites showed no BOP, PD≤3mm, and CAL of ≤2mm.

To collect the subgingival plaque, sterile gauze was used to isolate and dry the site to be sampled. Any supragingival plaque at the sampling site was removed with a cotton applicator. A sterile dental curette was inserted to the depth of the gingival sulcus, and a subgingival plaque sample was retrieved. Each sample was placed in a sterile labeled tube containing 50 µL TE buffer and stored in -80^{o} C for future analysis.

After that, interdental papillae at the same predetermined site were removed during periodontal surgeries. Briefly, after infiltration with an appropriate local anesthetic, submarginal incisions were performed and the mucoperiosteal flap was reflected. The portion of selected interproximal gingival papillae were carefully excised. The gingival samples then were trisected longitudinally the way that included both epithelial and connective tissue layers to generate samples for placement into sample vials: two deep-frozen in liquid nitrogen for total tissue proteins and RNA analysis (data not shown in this this thesis). The third piece was fixed in 10% formalin for immunohistochemistry (IHC). At least 2 diseased papillae were collected in each patient, and whenever available, healthy periodontal tissue was harvested from an adjacent site.

Peripheral blood samples were collected for the study of anti-inflammatory effect of bromodomain inhibitor, which is not presented in this thesis.

IHC

IHC was performed to determine the localization of histone marks in gingival tissues. Fixed gingival samples then embedded in paraffin and cut into 4-µm-thick serial sagittal sections. The sections deparaffinized, rehydrated, and pretreated with citrate buffer (10 mM sodium citrate, pH 6.0). Endogenous peroxidase activity disrupted with 3% H_2O_2. Non-specific protein binding was blocked with 5% goat serum in Antibody diluent (Dako) for 1h. Commercially available recombinant Anti-Histone H3 (acetyl K4+K9+K14+K18+K23+K27) antibody [RM1045] (Abcam, ab300641) then applied overnight at 4 ^oC (1:100 dilution). Sections were washed in 0.05% Tween 20 in phosphate-buffered saline (PBST) and incubated with biotinylated goat anti-rabbit secondary antibody. The signal was amplified with ABC reagent for peroxidase (Vector Laboratories) and detected with 3,30-diaminobenzidine (Vector Laboratories). Negative controls that omitted the primary antibody were run in parallel. The staining intensity and distribution were determined by light microscopy. Hematoxylin and eosin (H&E) staining was also performed.

IHC Scoring

For IHC staining, two well-calibrated examiners reviewed and scored the representative slides. Staining on connective tissues were scored based on the area and the intensity of the staining.

a. Percentage for Connective Tissue (A):

This criterion was to estimate the number of areas in the IHC stained tissue that show a positive signal in the respective connective tissue area. The connective area was first identified by the cell density, background staining or from H&E staining. The percentage of the positive nuclear staining area in total connective tissue was determined. The

following scoring system was used: Score 0 represented no positive staining; Score 1 represented that less than 30% of area in the connective tissue showed positive staining; Scores 2 and 3 represented 30-60% and >60% of cells showed positive staining, respectively.

b. Intensity of Staining for Connective and Epithelial tissue (B):

The intensity of the positive signaling was determined by observing the contrast of the signal to its background in its respective area on the tissue and evaluated on a scale from 0 to 3 respectively. A score of 0 represented a sample with no apparent signaling in the area of the tissue being evaluated. A score of 1 represented a sample with weak signaling that appears light and hard to contrast from the background. A score of 2 represented samples with apparent and moderate signaling in the signaling cells. A score of 3 represented samples with very obvious, contrasting, and strong signaling in the signaling cells of the area under evaluation.

c. Total IHC score

Total IHC score was calculated by A x B.

Histological Phenotype (Inflamed vs. Non-inflamed)

Further analyses were performed by determining the histological phenotype of each sample. Tissues with less than 20% area of immune cell infiltration were deemed as non-inflamed. Tissues with more than 20% area of immune cell infiltration were categorized as inflamed.

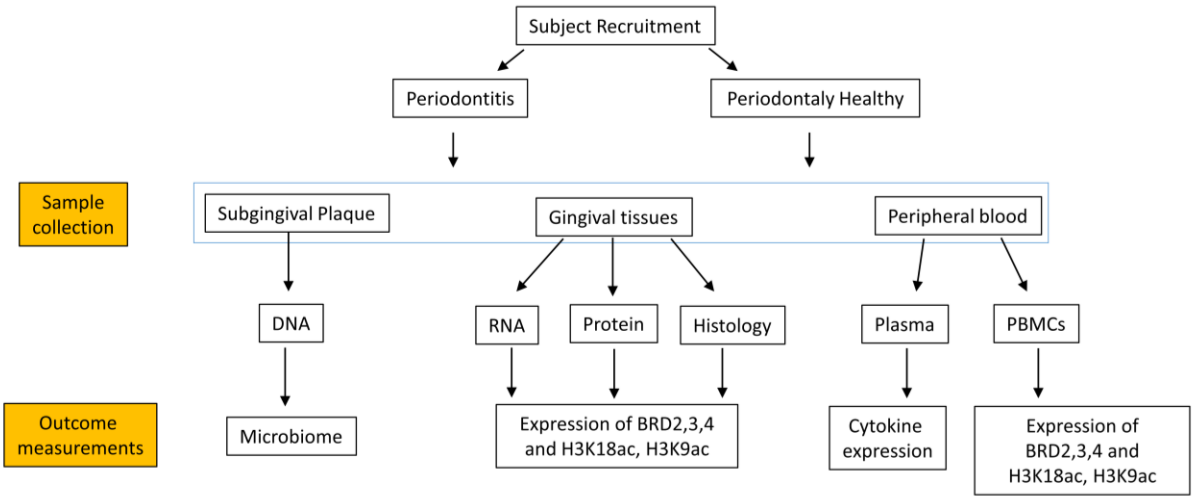
16S RNA Sequencing of Subgingival Microbiome

Bacterial genomic DNA was isolated using a genomic DNA isolation kit. Illumina DNA sequence libraries were prepared after the amplification of the V3-V4 region of 16S rRNA with the index barcodes. More than 30,000 sequence reads per sample sequence depth were obtained by Illumina MiSeq sequencer using the 2 X 300 bp format. The raw sequence reads were filtered by an in-house protocol to collect high-quality sequences. The operational taxonomic unit (OTU) of high-quality sequences were determined by the Ribosomal Database Project (RDP) classifier. The bacterial population and histone marks were clustered using Hierarchical Clustering (HCL) based on comparison and statistical analysis of the microbiome abundances between the different histone marks. Predominant taxa related to specific epigenetic markers were then identified. All published 16S rRNA gene sequences of oral microbiome were downloaded from Human Oral Microbiome Database (HOMD) and formed a 16S rRNA database. Our collected bacterial sequences from patients were searched against the 16S rRNA database using BLASTN. The associated taxa for the best-matched sequences were collected. The patient-related bacterial species and genus were analyzed based on sequence abundances.

Statistical analysis

A Power analysis was performed based on a previous study assessing the expression of an inflammatory marker in periodontitis and 100 subjects in each group are needed. In this thesis, we reported the results of first 39 subjects. For microbiome study, samples from the first 29 subjects were analyzed. Data were populated into GraphPad and Student's T-test was performed. P- value <0.05 was considered to be significant.

Figure 1. Study design.



Results

Results of the first 39 subjects (17 males and 22 female) were reported here. 24 patients were included in the diseased group with mean age of 53.5 years (ranging 40 to 74) and 15 in the healthy group with the mean age of 29 years (ranging 21 to 73). There were 2 patients with diabetes (both in the disease group) and the diabetic condition was well-controlled. There was no intergroup statistical difference in the race, site distribution (maxillae vs mandible) and smoking status between the two groups, however, more females were in the healthy group, and they were younger. (Table 2)

Total H3 acetylation in gingival tissue: Periodontitis vs. Healthy Samples

In the 39 samples, we were able to perform histological staining on 37 of them. 2 samples in the healthy groups were lost during embedding or sectioning. The Histone 3 acetylation (K4+9+14+18+23+27) was detected in the nucleus of gingival epithelial cells, fibroblasts, endothelial cells and infiltration immune cells (Figure 2). In epithelium, there was no significant difference between the periodontitis group and healthy group. In the connective tissues, higher percentage score (more area with positive staining) was seen in periodontitis samples compared to healthy tissues, however, it is not statistically significant ($p=0.0521$) (Figure 3). There was no difference in the intensity score and total IHC score (Figure 3).

Total H3 acetylation in gingival tissue: Inflamed vs. Non-inflamed Tissues

We found that there was a large variance at the inflammatory cell infiltration level among the samples. For example, some tissues from periodontitis patients had very limited inflammatory cell infiltration but some samples from healthy subjects showed significant cell infiltration

(Figure 4). Therefore, we classified the samples by their inflammation status (the extent of immune cell infiltration). In total, in the periodontitis group, 5 of the 24 tissues were classified as non-inflamed and in healthy group, 4 of the13 tissues were deemed as inflamed. Based on the histological phenotype, we found that the percentage score of acetylated H3 in inflamed tissues was dramatically higher than that in non-inflamed tissues, as well as the total IHC score (Figure 5).

Periodontal microbiota analysis

We examined the oral microbiome in the 28 samples (17 disease and 11 health patients) using next generation sequencing. 515 species were identified, belonging to 122 genus. 45 out of these species have >5% abundance in at least one of the 28 samples. We further compared the abundance of each of the 45 species between healthy and periodontitis patients. We found that 9 species had significant differences in abundance between disease patients and healthy subjects. Seven species, including *Peptostreptococcus stomatis*, *Fusobacterium sp. HMT 203*, *Prevotella intermedia*, *Fusobacterium sp. HMT 370*, *Fusobacterium nucleatum subsp. animalis*, *Alloprevotella tannerae*, and *Porphyromonas gingivalis* were more significantly abundant in disease patients while two species, *Veillonella atypica* and *Streptococcus sanguinis* were more abundant in healthy subjects. (Figure 6)

Table 2. Demographic summary of study subjects.

	Periodontitis (n=24)	Healthy (n=15)	P value
Sex			0.0522
Male	15	2	
Female	19	13	
Age			<0.0001
Range	40-74	21-73	
Median	53.5	29	
Race			
Caucasian	12	8	>0.9999
African American	10	7	>0.9999
Hispanic	2	0	0.5142
Asian	0	0	--
Sample site			0.7397
Max	14	10	
Mand	10	5	
Diabetic status			0.05142
Diabetic	2	0	
Non-diabetic	22	15	
Smoking status			>0.9999
Smoker / former smoker	6	4	
Non-smoker	18	11	

Figure 2. Representative histological images for the expression of H3K(4+9+14+18+23+27)ac in healthy and periodontitis tissues. The top panels were IHC staining and the bottom panels were H&E staining. PD: periodontitis.

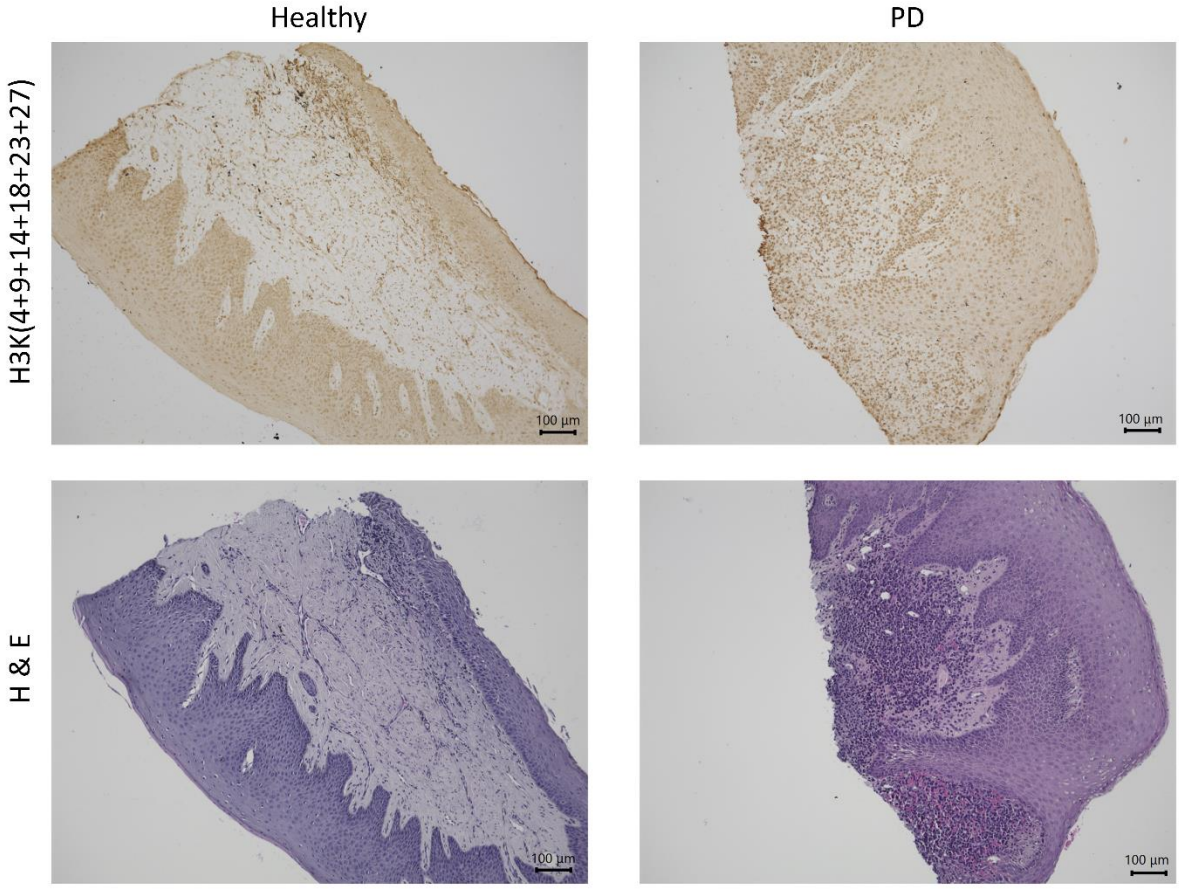


Figure 3. IHC scoring of H3K(4+9+14+18+23+27)ac staining based on clinical diagnosis (periodontitis vs. healthy groups). PD: periodontitis.

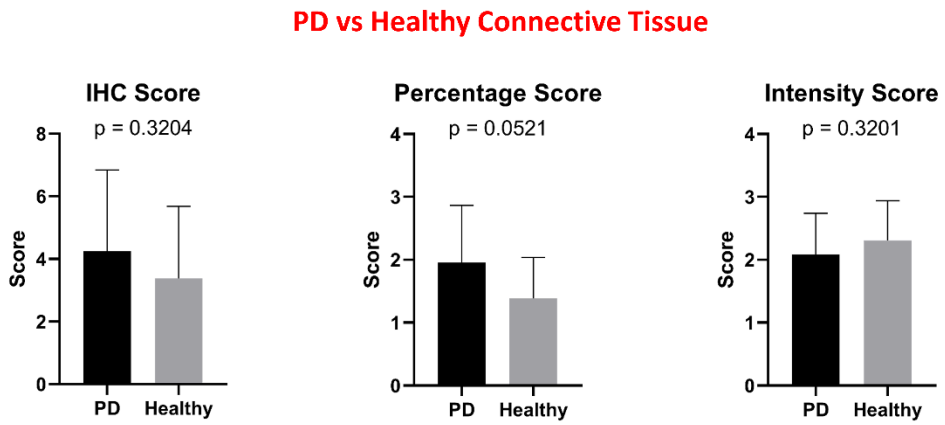


Figure 4. Different immune cell infiltration levels were seen in the samples. We found that there was a large variance at the inflammatory cell infiltration level among the samples. For example, some tissues from periodontitis patients had very limited inflammatory cell infiltration but some samples from healthy subjects showed significant cell infiltration. Therefore, we re-analyzed the samples by their inflammation status (the extent of immune cell infiltration). (PD: Periodontitis)

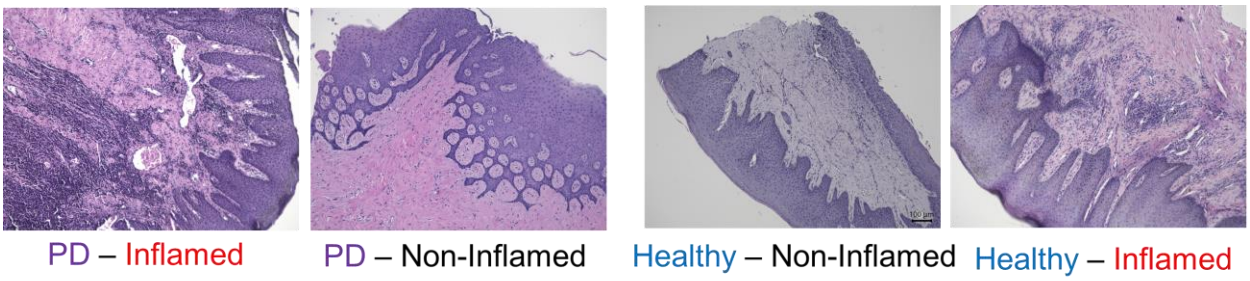


Figure 5. IHC scoring of H3K(4+9+14+18+23+27)ac staining based on histological phenotype (inflamed vs. non-inflamed groups).

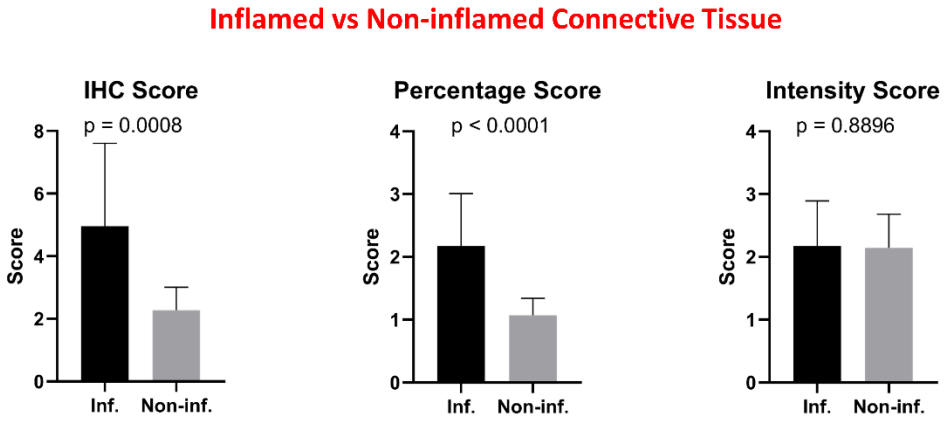
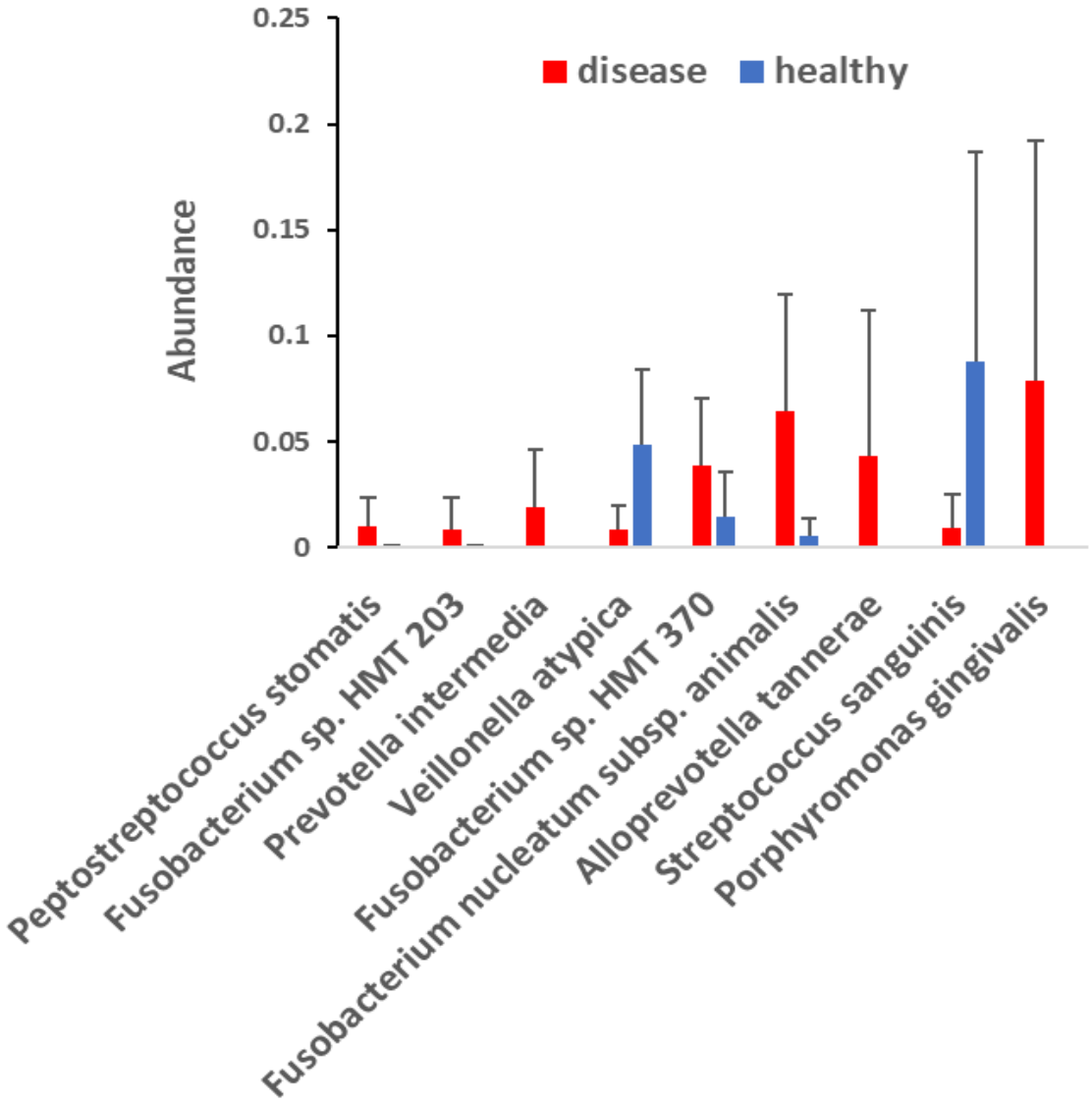


Figure 6. Subgingival microbiota analysis by 16s-Seq. Nine species showed significant difference (P<0.05) between periodontitis and healthy subjects.



Discussion

The overproduction of cytokines and matrix processing enzymes, and the pathological activation of osteoclasts are the results of a chronic inflammatory response in periodontitis, which often lead to irreversible tissue destruction and bone loss. [6] The activation or inactivation of inflammatory genes is regulated by epigenetic mechanisms. These mechanisms can alter by variety of stimuli, including systemic (such as smoking and diabetes mellitus) and local (such as bacteria and their virulence) factors, which alter the immunological responses. [14, 15,16] Acetylation of lysine residues removes the positive charges on histones, thereby decreases the binding to negatively charged phosphate groups of DNA. As a consequence, the condensed chromatin is converted into a loose structure that is associated with increased gene transcription. Upon microbial insults, host cells undergo massive changes in their transcription program to trigger an appropriate inflammatory response. It is not surprising that successful pathogens have developed specific mechanisms to manipulate the gene expression network in host cells, including histone acetylation, to favor their survival. In our study, we found that the acetylation level of H3 in the inflamed gingiva is dramatically higher than that in the non-inflamed tissues. This may be related to the massive immune cell infiltration in the local area and these cells have a high histone acetylation level because of their active inflammatory gene transcription. Rabbit polyclonal antibodies to acetylated H3 were used in our study, which can recognize multiple lysine sites including K9, K14, K18, K23 and K27. Further studies is needed in the future to dissect out which acetylated lysine sites are the key players mediating the inflammatory response in periodontitis.

We observed a high level of positive staining of acetylated H3 in gingival epithelial cells, however, there was no significant difference between periodontitis and healthy patients, nor between inflamed and non-inflamed tissues. Interestingly, other groups have reported that *Porphyromonas gingivalis* stimulated the H3 acetylation in human gingival epithelial cells. [12, 17,18] Martins et al. also showed that H3K9 is hyper-acetylated in the gingival epithelial tissues in murine experimental periodontitis. [19] The difference between our results and that from previous studies may due to the sensitivity of the IHC staining and our small sample size. Because the strong staining of acetylated H3 in our slides, it is difficult to observe any difference if that is rather subtle. More sensitive techniques such as immunofluorescence staining will be needed. It is also worth mentioning that pocket epithelium was not always present in our samples, which may be related to the embedding orientation and the severity of the disease. In the future, it would be valuable to analyze the H3 acetylation in samples with intact pocket epithelium.

The fact that histone acetylation is upregulated in periodontal inflamed tissues suggests that histone acetylation can be a target for treating periodontitis. The status of histone acetylation is usually "translated" by bromodomain and extraterminal domain (BET) proteins, a protein family characterized by two tandem bromodomains (BD1 and BD2) followed by an extraterminal domain. Intraperitoneal injection of JQ1, a pan-bromodomain inhibitor that targets both BD1 and BD2 of BET proteins, into mice inhibited inflammatory cytokine expression and prevented the experimental periodontitis induced by ligature ligation [20]. We also found that RVX-208, an epigenetic medication that specifically targets the second bromodomain of BET protein, can inhibit periodontal inflammatory response induced by *Porphyromonas gingivalis* and prevent alveolar bone in rats.

There are some limitations to our study. The relatively small sample size reduced the statistical power of our study, but we believe that this could be overcame if more subjects are enrolled. Another limitation of study is that some of the tissue samples did not reflect their disease diagnoses. For example, about 20% of the samples in periodontitis group did not have representative immune cell infiltration and 4 of the 13 samples in the healthy group demonstrated significant amount of immune cell inflation. This may be related to the specific location of the sectioning. It is also possible that the temporary crown and cement in the healthy patient irritated the gingiva that resulted in local inflammation. Therefore, to overcome this limitation, we regrouped the samples based on histological diagnosis (inflamed or non-inflamed). In the future, we recommend harvesting the tissue samples from the exact inflamed interproximal papilla and avoiding the buccal or lingual collar tissues. For the healthy group, we prefer tissues in the healthy site adjacent to tooth that needs crown lengthening.

Through 16s RNA sequencing, we identified a group of bacteria that had a higher abundance in diseased periodontal pockets and a couple of species that were more common in healthy sites. Some of them have been associated with periodontitis, such as *Prevotella intermedia* and *Porphyromonas gingivali.*[21, 22] However, *Alloprevotella tannerae* has not been related to periodontitis. *Fusobacterium sp. HMT 203* and *HMT-370* has be shown to be abundant in sites with peri-implantitis. [23] In the future, it will be interesting to further study the roles of these less studied species in periodontitis. More research is needed to determine how the host tissues' epigenetic regulation interacts with the periodontal microbiota.

Overall, our findings suggest that H3 acetylation level is increased in inflamed periodontal tissues. Future research to examine the biological role of certain histone modifications in periodontitis would greatly benefit from the data gathered from this study. The findings of this

study will also aid in the design of clinical trials to test the first "epi medication" for the treatment of periodontal diseases.

Conclusion

This study evaluated the histone acylation pattern of inflamed tissue in periodontal disease, particularly histone 3. Within the limitations of this study, our result suggests that a higher level of histone 3 acetylation was present in the inflamed gingival tissues compared to non-inflamed tissues. Our result suggests that histone acetylation may be a potential pharmacological target for treating periodontitis using epigenetic approaches in the future.

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