# 16S analysis of the subgingival biofilm and cytokine profile in patients receiving fixed orthodontic treatment

Thesis

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By

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## Abstract

**Introduction:** Microorganisms normally colonize the periodontal tissues, forming a biofilm, and changes in this biofilm can lead to colonization by opportunistic pathogens and lead to periodontal disease. Although we know that orthodontic appliances increase plaque retention, there are limited reports in the literature on how tooth movement affects the subgingival biofilm. The pressure tension theory states that orthodontic force causes pressure and tension sites in the periodontium, where there is bone resorption occurring in the pressure sites and bone formation occurring in the tension sites. The cytokine profile is also affected, as well as the amount of blood flow. Currently there are no studies describing whether the changes that occur during orthodontic tooth movement affect the composition of the subgingival biofilm, especially over the course of comprehensive orthodontic treatment, which lasts between one to two years for most patients.

**Objectives:** The aim of this study was to understand how orthodontic tooth movement affects subgingival biofilm development. The secondary aims were to examine whether there is any difference in subgingival biofilm and cytokine profile in anchor teeth vs. non-anchor teeth, and in pressure vs. tension sites.

**Methods:** Nine healthy subjects between the ages of 13 and 19 who were treatment planned for 1st maxillary premolar extractions were followed for 8 to 12 months. At each regularly scheduled adjustment appointment (once every 4 to 6 weeks), subgingival plaque samples and gingival crevicular fluid samples were taken from the mesiofacial and distofacial sites of the upper right canine (pressure and tension sites) and upper right first/second molar (anchor sites). Probing pocket depths, plaque index, and gingival index were recorded for the same teeth. Bacteria present in the samples were identified using 16S sequencing (Illumina MiSeq). Cytokine analysis was completed using the V-PLEX Proinflammatory Panel 1 Human Kit.

**Results:** 16S sequencing analysis is still incomplete. Almost all the cytokines and time points displayed no significant difference between the pressure, tension, and anchor sites. IFN-γ showed a significant difference at time point 9.5 months between the pressure and tension sites ($p=0.0385$). IL-12p70 also showed a significant difference at time point 5.4 months between the pressure and anchor sites ($p=0.0336$). TNF-α also showed a significant difference at time point 5.4 between the pressure and anchor sites ($p=0.0434$).

**Conclusions:** Cytokine analysis showed little statistically significant difference between pressure, tension, and anchor sites. Changes in concentration fluctuated around baseline over time, with IL-1β showing 2-4 month cycling of cytokine levels. These findings suggest that the oral cavity is resilient and can adapt to the burden brought upon by orthodontic forces without significant detrimental or permanent effects.

## Dedication

Dedicated to my wonderful husband. Thank you for believing in me and your unwavering support.

**Acknowledgments**

This project would not have been possible without the assistance of all those around me. I would like to thank my advisor, Dr. Purnima Kumar, BDS, MS, PhD, for her guidance and her passion for research; she has been my inspiration over the many years I've worked with her. I'm extremely grateful to have been able to work closely with her and learn from her wealth of experience. She has been a wonderful advisor and mentor.

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## Vita

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### Publications

Meng, Hsiu-Wan, Esther Chien, and Hua-Hong Chien. "Dental implant bioactive surface modifications and their effects on osseointegration: a review." Biomarker Research 4.1 (2016): n. pag. Web.

### Fields of Study

Major Field: Dentistry

Specialty: Orthodontics

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# Chapter 1. Introduction

Orthodontic treatment is so prevalent today that to many children and adolescents, it is a normal part of growing up. Having straight teeth and an ideal smile can increase quality of life, making it easier to find a partner[1], land a job interview[1], or have higher self-esteem[2]. Today, more adults are also seeking orthodontic treatment, and the American Association of Orthodontics (AAO) estimates that one in four orthodontic patients is an adult. The psychosocial benefits are well-established, but for many people, orthodontic treatment will not significantly improve dental function or health. Though straighter teeth may be easier to clean, good oral hygiene appears to be determined more by the motivation of the patient rather than how straight their teeth are.[3] But dental health is important for orthodontic treatment success. If a patient who has active periodontal disease undergoes orthodontic treatment, the periodontal problem is exacerbated, the patient will lose bone at a much faster rate, escalating the risk of losing teeth due to lack of a good periodontal support.

## Periodontal disease

Periodontal disease was originally believed to be caused by specific bacteria, but now research has shown that it is an inflammatory disease that is associated with dysbiosis, or disruption of the healthy microbial homeostasis. It can result from

unfavorable interaction among three factors: bacterial infection, the host's immunological reaction, and the environment.

### Bacterial infection

The presence of bacteria is necessary for periodontitis to develop. Several hypotheses have been developed and changed throughout the years. Early on, the nonspecific plaque hypothesis was proposed because an association was found between the amount of plaque and the presence of disease. Tissue destruction was due to the production of toxic byproducts, which the host would be unable to neutralize if the amount was too much. It was found that some individuals had large amounts of plaque but did not develop periodontitis, and so the specific plaque hypothesis was proposed. This hypothesis states that certain bacteria are more pathogenic than others and led to the study and identification of periodontal pathogens.[4]

There are many well-known periodontal pathogens. They have traditionally been categorized into the red complex (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*), the orange complex (*Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigrescens*, *Parviomonas micra*, *Eubacterium nodatum*, and various *Campylobacter* species), and yellow complex (*Streptococcus intermedius*, *Streptococcus sanquinis*, *Streptococcus oralis*, *Streptococcus mitis*, and *Streptococcus gordonii*), with the presence of large numbers of red complex bacteria indicating the highest risk of having periodontitis.[5] But this hypothesis also failed to explain the progression of gingivitis to periodontitis, since periodontal pathogens could also be found

in healthy individuals. The current understanding of periodontitis is explained by the ecological plaque hypothesis, which states that the host response (inflammation) is what decides which microbes thrive, and the biofilm and the host are in balance. When this balance is broken (e.g. due to excessive amounts of plaque, systemic diseases, environmental factors), the changes in host response can lead to dysbiosis of the microbial community, resulting in bone loss.[4] With this view that periodontal disease is caused by a shift in the microbial community to an unbalanced and unhealthy biofilm, being able to track and map out whole microbiome changes may be more helpful than following only a few target species. With the advent of DNA sequencing, this has now become possible.

Before DNA sequencing, bacteria were identified through culturing. Subgingival plaque samples were taken and then cultured on various media. To quantify the bacteria, the number of resulting colonies were counted. To identify the bacteria, its morphology was described and tests such as the Gram stain and production of catalase could help narrow down the species.[6] The limitations are many: the bacterium in question has to be cultivatable, anaerobic bacteria needed to be transported very carefully, there's risk of contamination, and it requires a lot of time and resources.

The oral cavity is home to an estimated 750 species.[4] DNA sequencing allows us to identify all the bacteria that are present in the sample, so it is possible to examine changes in the overall microbiome. 16S ribosomal RNA is found across all bacterial lineages and is highly conserved, and thus it is widely used in bacterial taxonomy studies.

Illumina MiSeq is one of the most widely used next generation sequencing platforms due to its cost-effectiveness and rapid turnaround time (chapter 12, Rupesh kanchi ravi).

The microbiome can be described based on diversity. (Dabdoub) Previous studies have shown that as a subgingival site progresses from health to disease, the microbiome becomes more diverse because new bacteria are now able to survive in that area. A healthy microbial environment also has a strong core microbiome, a group of microbes found commonly in all healthy individuals. When disease is present, the core microbiome becomes overrun by other more pathogenic microbes.[7]

**Host susceptibility**

Although bacteria play a large role, a susceptible host is also needed for periodontal disease to develop. The classic paper by Löe et al (Experimental gingivitis in man) that found that gingivitis developed in every subject within 14 days without any form of oral hygiene illustrates how gingivitis is directly linked to the presence of bacteria.[8] But why gingivitis progresses into periodontitis and bone loss is less clear. There is more and more evidence to support the hypothesis that the host inflammatory and immune responses, which are influenced by genetics, stress, diet, and general health, may be more important in determining whether or not gingivitis progresses into a more severe form of disease.[4]

### Environmental factors

Two established risk factors for periodontal disease are cigarette smoking[9, 10] and diabetes[9]. Orthodontic appliances appear to cause only transient changes in the supporting soft tissue, such as gingivitis and gingival hyperplasia. Unless there is existing disease, most studies have found that pocket depths and gingival health return to baseline normal a few months after appliance removal.[11-14]

## Cytokines

Cytokines are small proteins involved in cell signaling. They can be both pro-inflammatory and anti-inflammatory.[15] IL-1β, IL-2, IL-5, IL-6, IL-8, TNF-α, and IFN-γ upregulate inflammatory reactions such as vasodilation and tissue invasion by leukocytes. IL-4 and IL-10 are anti-inflammatory cytokines.[16]

Table 1. Pro-inflammatory cytokines

| | | |
|---|---|---|
| IL1-β | Increases production of other pro-inflammatory mediators (PGE2, IL-6); stimulates neutrophil activity | Key role in the pathogenic processes; enhances alveolar bone-resorption; fuels the inflammatory reaction |
| IL-6 | Regulates cell growth and differentiation: osteoblasts, B and T lymphocytes | Impairs osteoblast growth and function; increases osteoclast formation from monocytes |
| IL-8 | Involved in recruitment of neutrophils and improving RANKL expression | Increases osteoclast production and activation |
| TNF-α | Major regulator of immune cells' activity; involved in the acute phase reaction | Stimulates damage (by osteoclasts) and prevents repair of periodontal tissues (by fibroblast death); starts IL-1β, $PGE_2$ synthesis |

Table adapted from Surlin et al.[17]

Table 2. Anti-inflammatory cytokines

| | | |
|---|---|---|
| IL-10 | Decreases cytokine production by immune cells; reduces inflammatory response | Down-regulates periodontal inflammation by reducing cytokine synthesis in immune cells |
| IL-4 | Stimulates tissue repair and regulates immunity; regulates differentiation of Th2 cells | Decreases production of Th2 cells, with important implications to periodontal damage |

Table adapted from Surlin et al.[17]

Cytokines are involved during orthodontic tooth movement. IL-1β, IL-6, and TNF-α initiate the process of bone resorption, through the regulation of RANKL and OPG. RANKL binds to RANK receptors on osteoclasts to activate them, tipping the scale towards osteoclastogenesis and increased bone resorption. OPG is a natural inhibitor because it can bind to RANKL, preventing it from binding to RANK receptors.[18]

Studies have found that levels of IL-1β increased at sites of tension and compression after placement of elastic separators mesial to the 1st molars.[16, 19, 20] The same thing was found after distal movement of maxillary 1st premolars after 2nd premolars were extracted[16, 21] as well as within 1 day of application of force to distalize canines.[16, 22, 23] One study also found that IL-1β levels correlated to speed of tooth movement.[23]

IL-8 was found to increase after 4 days[24] but decrease to below baseline levels after 7 days.[16, 25] TNF-α and IL-6 were found to increase within 1 day of force application.[16, 21]

Grant et al[16] followed 20 patients during canine retraction using 100 g NiTi coil springs. The 2nd molars were used as controls. Canine movement was measured using digital calipers on models. Both right and left canine and molar were sampled at the distopalatal (compression) and mesiobuccal (tension) sites using Periopaper strips. Samples were collected before commencement of treatment, 3 months into treatment before application of distalizing force but after placement of archwires, and 4 hours, 7 days, and 42 days after distalizing force was applied to the maxillary teeth. They looked at 10 cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α, and GM-CSF.

IL-2, IL-4, IL-5 and IL-10 were below the limits of detection. Four hours after force application, cytokine levels were raised significantly for IL-1β, IL-8, and TNF-α at tension sites and for IL-1β and IL-8 at compression sites. The 3 cytokines mentioned remained raised after 42 days at tension sites. The 2nd molars showed significant increases in GM-CSF and TNF-α 4 hours after force application only.

The authors also found that IL-1β quantities were higher 3 months into treatment (before distalizing force), and 4 hours, 7 days, and 42 days after distalizing force was applied at the tension sites. At compression sites, IL-1β concentration was higher after 42 days. IL-6 and IFN-γ had significantly higher quantities 4 hours after force application at tension sites. TNF-α had significantly greater quantities at 3 months into treatment (before application of distalizing force) and 4 hours after force application at compression sites.

**Involvement in periodontal disease**

As mentioned previously, the host response is also involved in the development of periodontitis. When cytokines are not regulated properly, periodontal disease can develop or worsen more rapidly.[26] Elevated concentrations of TNF-α, IL-6, IL1-β and IFN-γ have been associated with periodontal lesions and disease activity.[17]

IL1-β upregulates matrix metalloproteinases and downregulates tissue inhibitors of metalloproteinase production and stimulates bone resorption and is known to stimulate bone loss and inhibit bone formation. IL-6 is involved in hematopoiesis, acute phase response, and plays a role in the transition between acute and chronic inflammation. It

remains longer in the plasma compared to IL1-β and TNF-α so is used as a marker for inflammation. It is produced in inflamed tissues after cellular activation by bacterial lipopolysaccharides or cytokines such as IL-1β and TNF-α. TNF-α can stimulate production of secondary mediators to amplify the degree of inflammation.[27]

IL-10 is an anti-inflammatory cytokine that inhibits expression of proinflammatory cytokines. It upregulates recruitment and activation of B cells while downregulating T helper 1 cells and may contribute to controlling the progression of periodontal disease.[27]

**Involvement in orthodontic tooth movement**

After the application of orthodontic force, there is an aseptic acute inflammatory reaction that lasts for 1-2 days. This is followed by chronic inflammation which is associated with increased release of cytokines such as TNF-α, TNF-β, IL-1, PDGF, INF-γ and RANKL.[17]

During the inflammatory response triggered by tooth movement, cAMP levels increase in the first 4 hours in the PDL. In the first couple hours after orthodontic forces are applied to the teeth, prostaglandin and interleukin-1β levels also rise[3]. Inflammation transitions from an acute phase to a chronic phase after several days[28]. Changes in cytokines can be detected in the gingival crevicular fluid. In tension sites, IL-1β, TNF-α, MMP-9, and TIMPs 1 and 2 levels are significantly higher within 4 hours to 42 days after orthodontic forces are applied to teeth. In compression sites, IL-1β and IL-8 levels are

significantly higher after 4 hours, MMP-9 after 7 days until 42 days, and RANKL after 42 days[16].

IL1-β is a proinflammatory cytokine involved in acute phase inflammation. IL-6 is involved in formation of osteoclasts. IL-8 contributes to neutrophil recruitment and improving RANKL expression, activating osteoclasts and increasing their production. TNF-α induces osteoclast differentiation. IFN-γ increases during late stages of orthodontic treatment and controls osteoclastogenesis. IL-4, IL-10, and IFNγ prevent resorption of bone.[17]

The difference in cytokine composition in tension versus compression sites may affect the biofilm in those sites as well, and so far there have been no studies linking the cytokine profile during tooth movement with the composition of the subgingival biofilm.

**Orthodontic treatment and periodontal health**

For a successful outcome, orthodontic treatment depends on good oral health, including periodontal health. There are several ways orthodontic tooth movement using fixed appliances can influence the subgingival microbiome and periodontal health.

Conventional braces exponentially increase the difficulty of keeping teeth clean, and changes in daily oral hygiene habits must be made to accommodate the presence of brackets and bands on teeth and wires in the mouth. Studies have shown that following the placement of braces, visible plaque and gingival inflammation increases significantly[29, 30].

Metal corrosion can be toxic to certain bacteria, influencing which species can survive. A study by Speer et al[31] even found that the number of periodontal pathogens decreased during orthodontic treatment, suggesting it was due to metal corrosion from the fixed orthodontic appliances.

Most patients who undergo orthodontic treatment are children or adolescents, who may be experiencing considerable hormonal changes. Hormone levels can also affect the microbial composition.

Tooth movement involves two events that can also potentially affect subgingival biofilm composition: inflammation and increases/decreases in blood flow. For tooth movement to occur, the surrounding periodontal ligament (PDL) and alveolar bone must remodel, which means inflammation of those tissues must also occur[32]. Periodontal disease is characterized by uncontrolled inflammation[33], and if orthodontic movement is attempted in the presence of uncontrolled inflammation, periodontal disease progresses much more rapidly.

**Pressure/Tension Theory of Tooth Movement**

Blood flow around the tooth is altered by orthodontic movement. As teeth move, there are areas of tension and compression in the bone and PDL. In compression areas, the blood flow is decreased or even halted depending on the amount of force used. Light forces compress the blood vessels and lead to frontal resorption, and osteoclasts can arrive in two waves: first from the PDL side of the tooth socket to start removing bone and second from areas farther away. Heavy forces occlude the blood vessels and lead to

undermining resorption, where the osteoclasts only arrive in one wave because they must be recruited from elsewhere and must start removing bone from the bony side of the tooth socket, which is composed of dense cortical bone. Sterile necrosis occurs during undermining resorption. On the tension side of the tooth, normal blood flow is maintained, or even increased, and osteoblasts form bone.[3] These changes in blood flow can potentially affect the biofilm composition due to changes in oxygen levels, nutrient availability, and presence of immune cells.

### Long-term periodontal effects of orthodontic tooth movement

There are several long-term studies that have found minimal changes in crestal alveolar bone levels in patients who had previous orthodontic.[11, 34] Others have found no difference between individuals who had a history of orthodontic treatment versus those who had no treatment, suggesting that bone loss is not related to previous orthodontic treatment.[11, 35-37]

Root resorption, which is an iatrogenic effect of orthodontic tooth movement, can occasionally cause significant shortening of roots. This means that if a patient develops periodontal disease later on, they are more at risk of losing that tooth, because losing the same amount of bone has a more significant effect when the root is shorter. But there is no documented case in the literature on tooth loss due to root resorption. (taric??)

Any association between previous orthodontic treatment and periodontal disease later in life appears to be because the same individuals who seek out orthodontic treatment are also more likely to see out periodontal care later on in life.[3]

### Does orthodontic treatment improve periodontal health?

It makes sense that teeth that are crowded are more difficult to clean, and thus malocclusion may contribute to poor oral hygiene and thus periodontal disease. But a more significant factor appears to be the individual's own motivation for good hygiene and oral health, whether or not they have straight teeth.[3, 38]

Occlusal trauma used to be considered a primary cause of periodontal disease, but now it is recognized to be only a secondary factor, if it is even associated at all with a periodontal lesion.[39]

Because tooth drift is associated with periodontal disease, it used to be thought that malocclusion contributed to the development of periodontitis, but now we know that drifting of teeth is actually due to periodontal attachment loss.[38, 39]

## Previous studies on subgingival microbiome changes during orthodontic treatment

There are several papers that have looked at the changes in the subgingival plaque in orthodontic patients. A recent systematic review by Lucchese et al[40] identified 51 studies which examined the entire oral microbiota, including samples of saliva, supragingival plaque, subgingival plaque, gingival crevicular fluid, mucosal swabs, and tongue swabs. Both removable and fixed appliances were allowed. The observation period ranged from before appliance placement to 3 months after appliance removal. Most studies were short-term and ended within 3 months of appliance placement. The subjects ranged in age from 4 years to 30 years old, with the majority of studies looking

at adolescents only. They found that orthodontic appliances increased the quantity of oral bacteria, and also affected the type of bacteria present, and changes could be seen as soon as 1 month into treatment. The use of orthodontic appliances was associated with an increase in gram-positive and gram-negative more aggressive bacteria associated with caries and periodontal disease. Removable appliances were found to have less of an impact compared to fixed appliances.

Another recent systematic review by Guo et al[41] found 13 studies, each following between 1 and 17 species of bacteria, and only included articles that studied subgingival plaque in patients undergoing fixed orthodontic treatment (bands and brackets). Most of the studies used PCR to analyze the microbes, a few cultured the bacteria, and one study used a radioactively marked DNA probe technique. All of the studies except one based the analysis on either the subject or the tooth, with only one study evaluating different tooth sites. There was a wide age range, including subjects 8 years old to 36 years old. The length of observation was before bracket placement up to 1 to 6 months of treatment time for most of the studies. A few looked at the weeks before and after bracket removal, and two studies evaluated the subjects before bracket placement up to several weeks to months after brackets were removed. Most studies reported an increase in the levels of periodontal pathogens after bracket placement before decreasing, and sometimes even returning to pretreatment levels a few months later.

An older systematic review by Freitas et al[42] included 4 articles, all of which were included in either the systematic review by Guo et al or Lucchese et al. All 4 studies looked at subgingival plaque and used culture methods to study the bacteria. The subjects

ranged from 12 to 22 years old, and the observation period ranged from 3 months to 3 years. They found that both the quality and quantity of bacteria were influenced by the presence of fixed appliances, and that it is a transitory phenomenon which may depend on oral hygiene.

There appears to be a gap in knowledge in how the entire subgingival microbiome might be changed over the course of fixed orthodontic tooth movement. A more comprehensive look at the changes that occur during orthodontic tooth movement and how they affect the composition of the subgingival biofilm is needed. A long period of observation is also needed, as orthodontic treatment lasts one to two years for most patients.

**Aims**

The aims of this study were 1) to understand how orthodontic tooth movement affects biofilm development; 2) investigate how orthodontic tooth movement affects biofilm development in anchor teeth vs. non-anchor teeth; 3) examine the difference in biofilm formation in tension vs. compression areas of a tooth that is being moved orthodontically.

**Hypothesis**

We hypothesize that there will be transitory changes in the subgingival microbiome with an increase in more anaerobic bacteria due to the increased amounts of

inflammation, plaque, and areas of less blood flow and lower levels of oxygen during orthodontic tooth movement using fixed functional appliances.

# Chapter 2. Materials and Methods

## Subjects

Ethical approval for the study was obtained from the Office of Responsible Research Practices at The Ohio State University (IRB approval number: 2019H0023). Patients were recruited from the Graduate Orthodontics Clinic and the Dental Faculty Practice at The Ohio State University College of Dentistry. Patients were treated by 7 different providers (6 residents and 1 faculty). Five faculty supervised the treatment of the subjects treated by the residents.

15 subjects between the ages 12 and 19 were enrolled in the study (4 female and 11 male) with an average age of 15 years old at the start of sample collection. Subjects were recruited between August 2019 and August 2020. Assent was obtained for subjects younger than 18 and consent was obtained from the subject's guardian. Consent was obtained for subjects 18 years and older.

Consecutive patients between the ages 10 and 21 who were treatment planned to have upper first premolars extracted for comprehensive orthodontic care were recruited for the study. A few declined to participate. To be included in the study, patients were required to be periodontally healthy, free of systematic disease (such as diabetes), a non-smoker, and not currently pregnant or lactating. They were required to be in permanent dentition (excluding 2$^{nd}$ and 3$^{rd}$ molars).

The goal was to follow the patients from before orthodontic treatment to 1 year after the start of orthodontic treatment.

**Sample collection**

Samples were collected before the start of orthodontic treatment and at each regularly scheduled adjustment up until 1 year into treatment. The collection sites were the mesiofacial and distofacial of #2 (if erupted) or #3 (the upper right 1$^{st}$ or 2$^{nd}$ permanent molar) and #6 (the upper right canine). The upper right quadrant was isolated using cotton rolls. 10 paper points were used to collect subgingival plaque from each site. 2 GCF strips were used to collect GCF from each site. Probing pocket depths, Plaque score, bleeding on probing (BOP), and gingival index (GI) were recorded for both teeth. Intraoral photos were taken at each appointment and the extraction space was measured using a periodontal probe. The samples were placed on ice during collection and then transferred to a -20°C freezer until ready for processing.

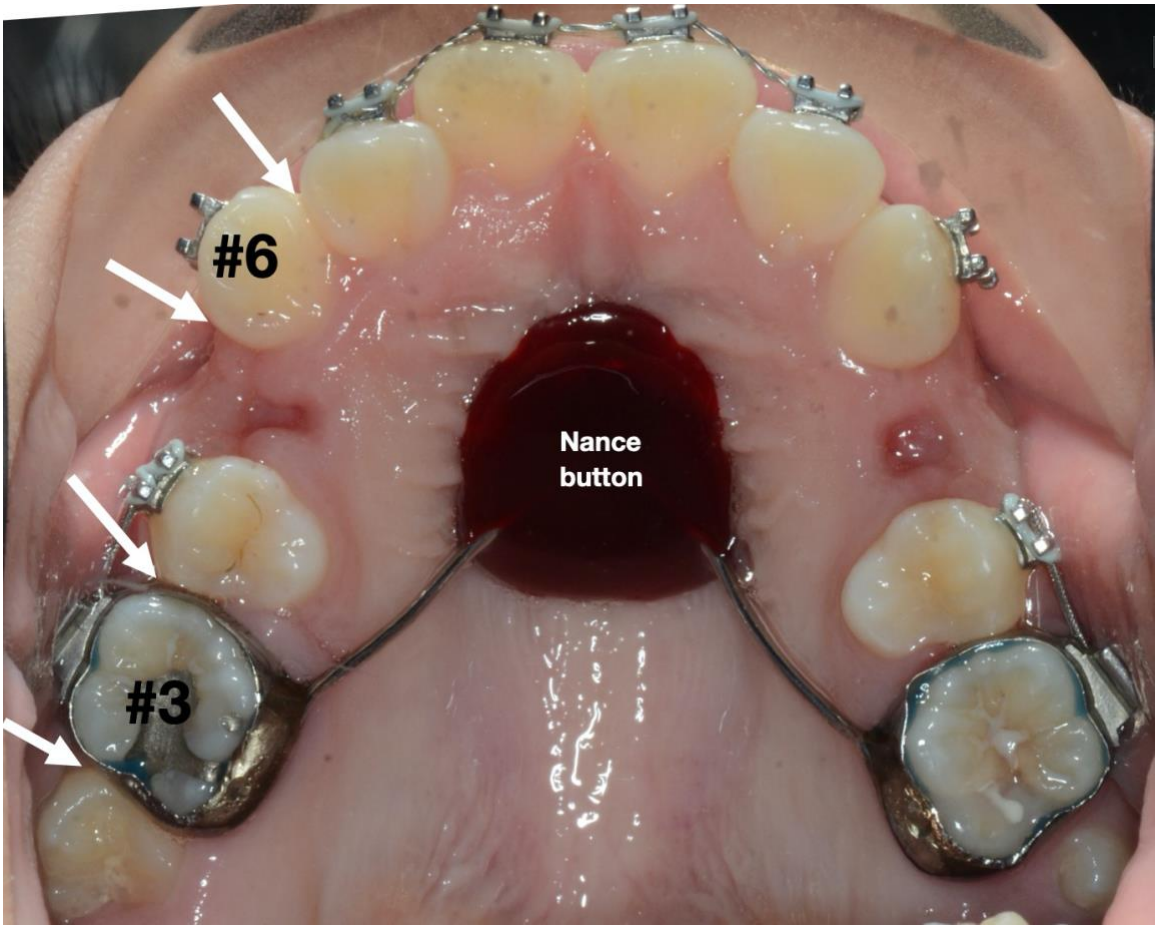


Figure 1. Example patient.

As the upper right 2nd molar was not fully erupted on this patient, samples were collected on the banded 1st molar. White arrows indicate the collection sites: mesial and distal of the canine and mesial and distal of the first molar. Many patients in the study had a Nance button as shown.

**DNA isolation and 16S sequencing**

DNA was isolated from the subgingival plaque samples using a Qiagen MiniAmp kit (Valencia, CA) according to the manufacturer's instructions.

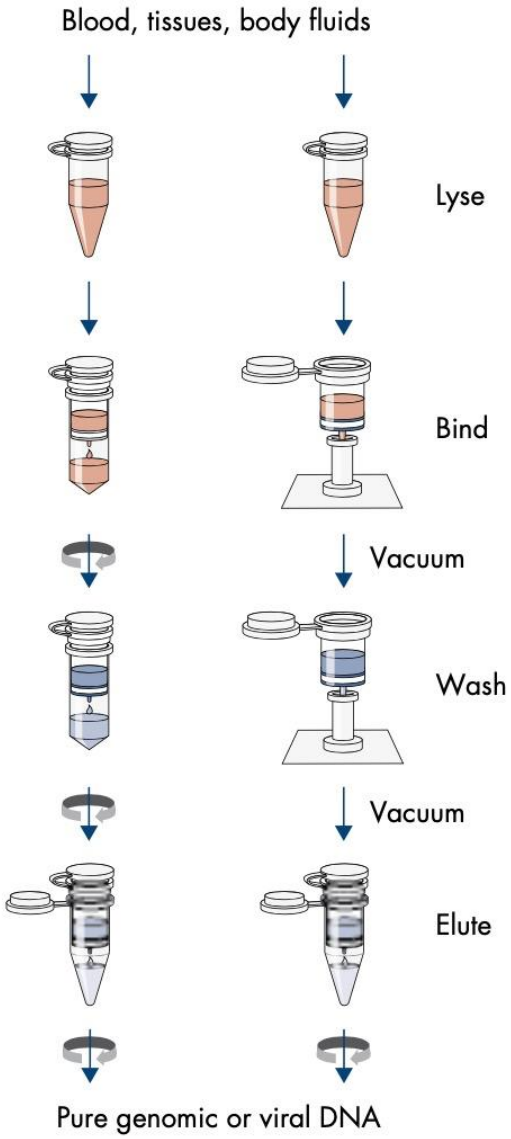


Figure 2. DNA isolation.

Taken from the manufacturer's guide. Illustrates how DNA is isolated using their kits. The cells are lysed, releasing DNA. Then the DNA is precipitated and bound to the column and washed. Finally, the DNA is dissolved and eluted from the column.

The isolated DNA was sent to the Molecular and Cellular Imaging Center in Wooster, Ohio for library preparation and 16S sequencing using Illumina MiSeq. Bacterial sequences from the V1-V3 region and V4-V5 region were identified. Raw reads with >10% unknown nucleotides or with >50% low quality nucleotides (quality value <20) were discarded.

The sequences were analyzed using the QIIME pipeline and PhyloToAST.[43, 44] First, adapter sequences, primers, and other unwanted sequences were removed using Cutadapt. Sickle was then used to trim 3'-end and sometimes 5'-end of reads as quality tends to be lower in those regions. To merge paired end reads, PEAR was used. Because multiple sequencing runs were performed in parallel, split_libaries.py in the QIIME pipeline was used to match the data with the samples. Chimera sequences (sequences generated from multiple transcripts or parent sequences) were removed. Operational taxonomic unit (OTU) picking against Human Oral Microbiome Database (HOMD) was completed. This step assigns sequences which are similar (at or above a threshold) to a taxonomic unit (e.g. a genus).

Two primers were used. Each primer detects a range of genera that the other does not detect, so together, recovery of a wider range of the microbiome is possible compared with a single primer alone. Some genera are picked up by both primers, so to prevent overcounting, the number of sequences assigned to an OTU by both primers was reduced by half. Primer averaging was carried out as previously described[45] using the implementation in the PhyloTOAST software suite.[44]

**Cytokine assay**

120 μL of 1X PBS was added to each sample and eluted. The samples were sent to the Clinical Research Center at the university and cytokine analysis was performed using the V-PLEX Proinflammatory Panel 1 Human Kit (Rockville, Maryland). Each sample was run in duplicate. Ten different cytokines were quantified: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α.

The kit comes with 96-well plates. The samples and a solution of detection antibodies conjugated with electrochemiluminescent labels are added to the wells, and analytes in the sample bind to antibodies that are immobilized on the working electrode surface in each well. Then the labeled antibodies bind to the analytes. A buffer for electrochemiluminescence is added, and the plates are read. The intensity of emitted light is proportional to the amount of analyte in the sample and concentration can be calculated based off of standard curves.

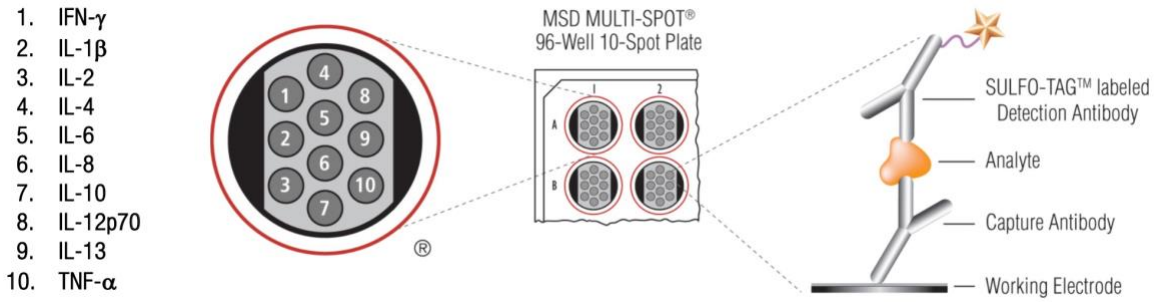


Figure 3. Cytokine analysis kit.

Taken from the Proinflammatory Panel 1 (human) kit insert by the manufacturer (MSD). This figure illustrates how the assay works. There can be multi-spot plates and small spot plates. The multi-spot plates can detect several different cytokines in a single well.

**Statistical analysis**

The analysis will use the tooth as a unit of measurement. Since each patient will contribute both moving and stable teeth, we will look into 1) the differences within each patient across time points for each of the two teeth 2) the differences between the two groups (teeth moved vs. not moved) across different patients.

**16S sequencing**

Analyses will be conducted using the QIIME[43] and PhyloToAST[44] pipelines. The sequences will be binned by sample and will be denoised using denoise_wrapper.py to reduce sequencing errors. All denoised sequences will be aggregated, and de novo operational taxonomic units (OTUs) will be identified. Sequences will be clustered into distinct OTUs at 99% similarity using the UCLUST method.[46] Chimeric sequences will be depleted using ChimeraSlayer (v. 1.9.0, identify_chimeric_seqs.py).[47] Sequences with an average quality score of 30 over a sliding window of 50 bp and length >200 bp will be assigned a taxonomic identity by alignment to the HOMD database using the Blast algorithm. Both phylogenetic (UniFrac) and non-phylogenetic (Bray–Curtis, Jaccard) distance matrices were utilized to estimate beta diversity. Non-metric dimensional scaling (NMDS) will be performed on distance matrices, and significance of clustering will be interrogated using Adonis with 999 permutations. NMDS plots will be generated by the R package Phyloseq and ggplot. Phylogenetic tree visualization will be created with iTOL (http://itol.embl.de/, version 3.4.1).

To investigate the effect of the different variables on the abundance of the OTUs, we will use generalized linear models for each OTU, and add the rate of movement, tension/compression, age, sex, and cytokine levels at explanatory factors (using mvabund and boral package in R - references).

**Cytokine analysis**

Statistics were run using JMP (SAS institute). 10 cytokines were analyzed: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. The change in cytokine concentration compared to time point 1 (baseline) was examined. The time points examined were: 1.2 months, 2.2 months, 3.5 months, 5.4 months, 8 months, 9.5 months, 10.9 months, and 12.2 months. Because patients returned at various point into treatment, the timepoints were averaged (e.g. the 1.2 month timepoint includes patients seen at 0.9 months up to 1.6 months).

The Tukey-Kramer HSD was used to evaluate differences between the pressure, tension, and anchor sites. Cytokine levels compared to baseline (before treatment) were graphed over time.

## Chapter 3. Manuscript

# Long-term cytokine profile of gingival crevicular fluid in patients receiving fixed orthodontic treatment

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## Abstract

**Introduction:** Inflammation is involved in both periodontal disease and orthodontic tooth movement, with cytokines playing an important role. Uncontrolled inflammation in periodontal disease results in bone loss, while aseptic inflammation in orthodontic tooth movement results in bone remodeling. There are numerous studies showing the immediate increase in cytokines such as IL-1β and TNF-α within 24 hours of force application, but what happens long-term is more ambiguous.

**Objectives:** The aim of this study was to understand how orthodontic tooth movement affects the cytokine profile in anchor teeth vs. non-anchor teeth and in pressure vs. tension sites over time.

**Methods:** Nine healthy subjects between the ages of 13 and 19 who were treatment planned for 1$^{st}$ maxillary premolar extractions were followed for 8 to 12 months. At each regularly scheduled adjustment appointment (once every 4 to 6 weeks), gingival crevicular fluid samples were taken from the mesiofacial and distofacial sites of the upper right canine (pressure and tension sites) and upper right first/second molar (anchor sites). Probing pocket depths, plaque index, and gingival index were recorded for the same teeth. Cytokine analysis was completed using the V-PLEX Proinflammatory Panel 1 Human Kit.

**Results:** Almost all the cytokines and time points displayed no significant difference between the pressure, tension, and anchor sites. IFN-γ showed a significant difference at time point 9.5 months between the pressure and tension sites ($p=0.0385$).

IL-12p70 also showed a significant difference at time point 5.4 months between the pressure and anchor sites ($p=0.0336$). TNF-α also showed a significant difference at time point 5.4 between the pressure and anchor sites ($p=0.0434$).

**Conclusions:** Cytokine analysis showed little statistically significant difference between pressure, tension, and anchor sites. Changes in concentration fluctuated around baseline over time, with IL-1β showing 2-4 month cycling of cytokine levels. These findings suggest that the oral cavity is resilient and can adapt to the burden brought upon by orthodontic forces without significant detrimental or permanent effects.

## INTRODUCTION

Orthodontic treatment is so prevalent today that to many children and adolescents, it is a normal part of growing up. Having straight teeth and an ideal smile can increase quality of life, making it easier to find a partner[1], land a job interview[1], or have higher self-esteem[2]. Today, more adults are also seeking orthodontic treatment, and the American Association of Orthodontics (AAO) estimates that one in four orthodontic patients is an adult.

Periodontal disease can result from unfavorable interaction among three factors: bacterial infection, the host's immunological reaction, and the environment. Uncontrolled inflammation is what eventually results in bone loss.

After the application of orthodontic force, there is an aseptic acute inflammatory reaction that lasts for 1-2 days. This is followed by chronic inflammation which is associated with increased release of cytokines such as TNF-α, TNF-β, IL-1, PDGF, INF-γ and RANKL.[17]

During the inflammatory response triggered by tooth movement, cAMP levels increase in the first 4 hours in the PDL. In the first couple hours after orthodontic forces are applied to the teeth, prostaglandin and interleukin-1β levels also rise[3]. Inflammation transitions from an acute phase to a chronic phase after several days[28]. Changes in cytokines can be detected in the gingival crevicular fluid. In tension sites, IL-1β, TNF-α, MMP-9, and TIMPs 1 and 2 levels are significantly higher within 4 hours to 42 days after orthodontic forces are applied to teeth. In compression sites, IL-1β and IL-8 levels are

significantly higher after 4 hours, MMP-9 after 7 days until 42 days, and RANKL after 42 days[16].

IL1-β is a proinflammatory cytokine involved in acute phase inflammation. IL-6 is involved in formation of osteoclasts. IL-8 contributes to neutrophil recruitment and improving RANKL expression, activating osteoclasts and increasing their production. TNF-α induces osteoclast differentiation. IFN-γ increases during late stages of orthodontic treatment and controls osteoclastogenesis. IL-4, IL-10, and IFNγ prevent resorption of bone.[17]

Grant et al[16] found that some cytokine levels differed between pressure and tension sites. A systematic review by Kapoor et al[48] found many studies showing the immediate increase in concentration of cytokines such as IL-1β and TNF-α within 24 hours of force application, but what happens long-term is more ambiguous.

The aim of this study was to understand how orthodontic tooth movement affects the cytokine profile in anchor teeth vs. non-anchor teeth and in pressure vs. tension sites over time.

## MATERIALS AND METHODS

### Ethics statement

This study was approved by the Office of Responsible Research Practices at The Ohio State University (Protocol number:2019H0023).

### Subject selection and recruitment

Informed consent (and assent from patients younger than 18) was obtained from 15 consecutive patients between the ages 10 and 21 at the Graduate Orthodontics Clinic and the Dental Faculty Practice at The Ohio State University College of Dentistry who were treatment planned to have upper first premolars extracted as part of comprehensive orthodontic care. Participants were required to be free of periodontal disease and systematic disease (such as diabetes), a non-smoker, not currently pregnant or lactating, and be in permanent dentition (excluding 2nd and 3rd molars).

**Sample collection**

Samples were collected before the start of treatment (if patients needed separators, before separators were placed) and at each regularly scheduled adjustment up to 1 year into treatment. Collection sites were the mesiofacial and distofacial of #2 (if erupted) or #3 (the upper right 1st or 2nd permanent molar) and #6 (the upper right canine). The upper right quadrant was isolated using cotton rolls. 10 paper points were used to collect subgingival plaque from each site. 2 GCF strips were used to collect GCF from each site. Probing pocket depths, plaque score, bleeding on probing (BOP), and gingival index (GI) were recorded for both teeth. The extraction space was measured using a periodontal probe. The samples were placed on ice during collection and then transferred to a -20°C freezer until ready for processing.

**RESULTS**

**Subject selection and recruitment**

Although 15 subjects were recruited, due to time limitations and broken appointments, only 6 patients were followed for the full year, 2 were followed for 10 months, and 1 was followed for 8 months. The remaining 6 were followed for less than 3 months and were not included in the data analysis.

The 9 patients included in the data analysis were treated by 5 residents, all in the same class, and supervised by 5 faculty. Five patients had Nance appliances. Two patients had RPE's at the beginning of treatment. One patient had a Herbst, and one patient had bonded upper first molars. Five patients were male and 4 were female. Two were African American, 3 were Caucasian, 3 were Hispanic, and 1 was African. At the start of treatment, they ranged in age from 13-19 years old.

**Cytokine analysis**

10 cytokines were analyzed: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. The change in cytokine concentration compared to time point 1 (baseline) was examined. The time points examined were: 1.2 months, 2.2 months, 3.5 months, 5.4 months, 8 months, 9.5 months, 10.9 months, and 12.2 months. Because patients returned at various point into treatment, the timepoints were averaged (e.g. the 1.2 month timepoint includes patients seen at 0.9 months up to 1.6 months).

Almost all the cytokines and time points displayed no significant difference between the pressure (canine-distal), tension (canine-mesial), and anchor (molar-mesial and molar-distal data pooled together) sites. Using the Tukey-Kramer HSD test, IFN-γ showed a significant difference at time point 9.5 months between the pressure and tension

sites (p=0.0385). IL-12p70 also showed a significant difference at time point 5.4 months between the pressure and anchor sites (p=0.0336). TNF-α also showed a significant difference at time point 5.4 between the pressure and anchor sites (p=0.0434).

Qualitative changes in concentration of each time point compared to baseline are shown in Figures 1-5. The cytokine levels appear to cycle around baseline, with increases and decreases throughout the observation period.

## DISCUSSION

A lot of research has been completed regarding the cytokine profile in health and disease (gingivitis and periodontitis), and during orthodontic tooth movement. A systematic review by Kapoor et al[48] found 39 articles and concluded that there was an immediate release of inflammatory cytokines involved in bone resorption such as IL-1β and TNFα. Ten studies found that peak levels of these cytokines occurred at 24 hours for continuous force application. Eight other studies reported a peak level anywhere between 4 hours to 6 months. Three studies found a decrease to baseline levels later on from 48 hours to 21 days. Two studies[57, 58] found that repeated activations when using interrupted force increases cytokine concentrations compared to continuous force, but the follow-up period was only 1-3 weeks. Significant heterogeneity was found among the studies, making it difficult to draw comparisons and conclusions.

A few longer-term studies warrant a more in-depth look. A study by Ren et al[21] (that was also included in the aforementioned systematic review) looked at concentration of IL-1β, TNF-α, IL-6, and IL-8 in GCF both short and long-term. 6 adult subjects participated in the short-term study. Separators were placed between the upper canines and upper first premolars for 24 hours. The maxillary 1$^{st}$ premolars were used as the experimental teeth and the antagonistic teeth were used as controls. 6 adolescent patients who needed extraction of maxillary 2$^{nd}$ premolars participated in the long-term study and 5 timepoints were examined: before tooth extraction and before orthodontic treatment (T0), after tooth extraction and initial alignment (T1); and 1, 2, and 3 months after

retraction of 1st premolars using light continuous force (T2, 3, 4). The authors did not describe how this force was applied.

For the short-term study, the authors found that TNF-α, IL-1β, and IL-6 levels were significantly increased at 24 hours, which was expected because they are part of the first wave of cytokines. For the long-term study, they did not find any statistically significant change in cytokine levels at all study points except IL-8 was elevated significantly at T1 and then decreased to baseline levels. IL-8 is a part of the second wave of cytokines. All cytokines tested returned to their baseline levels during retraction of the 1st premolars.

The authors concluded that when orthodontic force is applied, inflammatory cytokines are produced, triggering a cascade of cellular events, which then stabilizes over time until the next reactivation.

Başaran et al[25] (also included in the systematic review) looked at IL-2, IL-6, and IL-8 concentrations in GCF. They looked at 2 periods of orthodontic treatment: during leveling and during retraction. Samples were taken at baseline, 7 days, and 21 days into initial leveling of the teeth. Baseline samples for retraction were taken at 6 months, and then 7 and 21 days after starting retraction. They did not find any statistically significant differences except a decrease in the concentration of IL-8 on the 7th day of leveling.

Finally, Iwasaki et al[23, 59] (also included in Kapoor et al's systematic review) examined IL-1β levels in 7 patients undergoing maxillary 1st premolar extractions and distal movement of the maxillary canines over a period of 84 days. The mesial and distal site of the maxillary canine was used as the experimental sites, and a site at or near to a

mandibular canine was used as the control site. Day 0 was defined as baseline, which was the day canine retraction forces were initiated. All subjects received either a Nance or a Nance/TPA combination appliance. A calibrated spring was used for retraction. It appears that mandibular teeth were not bracketed during this time. They found that IL-1β levels increased within 3 days after loading, and then dropped to baseline by 14-28 days, and then continued to cycle about every 28 days, which the authors reported were similar to the results of Grieve et al[60] and Uematsu et al[61, 62] (all 3 articles were also included in Kapoor et al's systematic review).

Immediate and short-term changes were not examined in our study. Statistical analysis was only run comparing tension vs. pressure vs. anchor sites. Changes over time are shown qualitatively in graphs. Minimal difference was found between the 3 different sites. Several variables may explain the lack of significance. It appears that most studies found significant changes within 24 hours of force application, a time point not included in our study. Due to the number of different faculty and residents treating the patients, when and how force was applied to teeth greatly varied among the subjects. Many studies reported that cytokine levels returned to baseline levels over time, as the system stabilized, which could be another reason why we did not detect differences among the 3 sites. Our data also shows that cytokine levels appears to hover around baseline over long periods of time with periodic increases and decreases that do not show a clear linear pattern.

It does appear that our data for IL-1β displays some cycling as described by Iwasaki et al[23, 59], Grieve et al[60], and Uematsu et al[61, 62]. They reported a periodicity of 28

days, but in our data the increase and decrease in IL-1β concentration appears to range between 2-4 months.

The lack of a difference of cytokine levels among the pressure, tension, and anchor sites and insignificant change over longer follow-up periods found in our study and several others demonstrate the resilience of the oral cavity. It is encouraging to know that even under the burden of orthodontic tooth movement, when bone is under constant remodeling and there are unfamiliar appliances on the teeth and in the mouth, the oral cavity can adapt and stay healthy. Cytokine levels may be increased temporarily, but in the long-term over the course of treatment, they return back to baseline levels. This can also explain why orthodontic treatment doesn't appear to cause significant long-term periodontal damage (i.e. alveolar bone loss),[38] with many studies also finding improvement of inflammation and pseudopocketing after appliances are removed.[14, 49]

**Limitations**

There are several limitations to this study. The 2nd molar was sampled if it was erupted, otherwise the 1st molar was sampled. Most patients in the clinic do not have their 2nd molars bonded at the start of treatment, and none have the 2nd molars banded. Most of the patients who participated in the study had their 1st molars banded because they had a Nance appliance. There was one patient who had a crown-design Herbst on the 1st molars. There have been studies showing that plaque and clinical periodontal parameters worsen when bands and brackets are placed on teeth, and that there are also differences between teeth that are banded vs. teeth that are bonded.[50] It was also extremely difficult

to collect samples from the distofacial of the 2nd molar. Grant et al[16] took samples from the distolingual of the 2nd molars, that may have been an easier site to sample from compared to the distobuccal since the cheek would not have been in the way.

The treatment progress varied greatly among patients due to patient compliance (e.g. late to appointments, missed appointments) and different philosophies of the faculty and resident providing treatment. Most patients had steady progress for space closure and canine retraction, but one still had almost the full amount of extraction space present after 1 year into treatment.

There was no control group of subjects who did not undergo orthodontics at all. A split mouth design would not have been ethical because it would have delayed placing braces on either the other quadrant or the lower arch for 1 year, which would likely increase treatment time by the same amount of time and complicate treatment mechanics. It would be difficult to recruit patients of the same age not undergoing orthodontic treatment to sample every 4-8 weeks but can be attempted in a future study. A control group would be beneficial to rule out the effects of hormonal changes during adolescence, as the microbiome can also be influenced by changes in hormone level.

The residents whose patients were participating in the study were asked to not have their patients brush at the beginning of the appointment until samples were collected, but occasionally the patients were still allowed to brush beforehand. During Covid-19, hydrogen peroxide mouth rinse was required at the beginning of all appointments. Most of the time samples were collected before the patient rinsed, but there were 1-2 appointments where the patient rinsed before samples could be collected.

A larger sample size would is needed as only 6 patients had the full year of follow-up completed, 2 were followed for 10 months, and 1 was followed for 8 months.

One patient had a change in treatment plan mid-treatment and upper 2nd premolars were extracted instead of upper 1st premolars.

Reliability testing would have improved the quality of the study if tooth movement had been measured on models using digital calipers as done in several other studies. This would have added more time for the patient during the sampling process, which may not have been feasible due to interference with the clinic and resident's schedules. More frequent calibration of the researchers completing sample collection and clinical measurements or limitation of sample collection/clinical measurements to 1 researcher would have also been.

Finally, the clinic was closed for 3 months from March 2020 to June 2020, during which no samples were collected and no patients were seen for adjustments. This caused a gap in sample collection, which occurred during months 4-9.

## CONCLUSIONS

The levels of pro-inflammatory cytokines in the gingival crevicular fluid such as IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α appear to fluctuate around baseline over long periods of time, at times increasing and at times decreasing.

No difference was found among the pressure, tension, and anchor sites in terms of cytokine concentration at any time point for all cytokines examined except for IFN-γ at

9.5 months (decrease in concentration in pressure sites from baseline was significantly greater compared to tension sites), IL-12p70 at 5.4 months (decrease in concentration from baseline in pressure sites was significantly greater compared to anchor sites), and TNF-α at time point 5.4 months (decrease in concentration from baseline in pressure sites was significantly greater compared to anchor sites).

These findings suggest that the oral cavity is resilient and can adapt to the burden brought upon by orthodontic forces without significant detrimental or permanent effects.

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## FIGURE LEGENDS

**Figure 1. Change in concentration of IFN-γ and IL-1β over time.**

Change in concentration compared to baseline for IFN-γ and IL-1β. A positive number means the concentration increased and a negative number means the concentration decreased.

**Figure 2. Change in concentration of IL-2 and IL-4 over time.**

Change in concentration compared to baseline for IL-2 and IL-4. A positive number means the concentration increased and a negative number means the concentration decreased.

**Figure 3. Change in concentration of IL-6 and IL-8 over time.**

Change in concentration compared to baseline for IL-6 and IL-8. A positive number means the concentration increased and a negative number means the concentration decreased.

**Figure 4. Change in concentration of IL-10 and IL-12P70 over time.**

Change in concentration compared to baseline for IL-10 and IL-12p70. A positive number means the concentration increased and a negative number means the concentration decreased.

**Figure 5. Change in concentration of IL-13 and TNF-α over time.**

Change in concentration compared to baseline for IL-13 and TNF-α. A positive number means the concentration increased and a negative number means the concentration decreased.

# Chapter 4. Results

## Subjects

Although 15 subjects were recruited, due to time limitations and broken appointments, only 6 patients were followed for the full year, 2 were followed for 10 months, and 1 was followed for 8 months. The remaining 6 were followed for less than 3 months and were not included in the data analysis.

The 9 patients included in the data analysis were treated by 5 residents, all in the same class, and supervised by 5 faculty. Five patients had Nance appliances. Two patients had RPE's at the beginning of treatment. One patient had a Herbst, and one patient had bonded upper first molars. Five patients were male and 4 were female. Two were African American, 3 were Caucasian, 3 were Hispanic, and 1 was African. At the start of treatment, they ranged in age from 13-19 years old.

## 16S sequencing

Data analysis of the 16S sequencing could not be completed at this time due to delays from Covid-19 and also the transition to Workday at the university. 192 samples have been sequenced, but we are still waiting for the sequences of 98 samples, which we should receive in early March 2021.

## Cytokine analysis

10 cytokines were analyzed: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. The change in cytokine concentration compared to time point

1 (baseline) was examined. The time points examined were: 1.2 months, 2.2 months, 3.5 months, 5.4 months, 8 months, 9.5 months, 10.9 months, and 12.2 months. Because patients returned at various point into treatment, the timepoints were averaged (e.g. the 1.2 month timepoint includes patients seen at 0.9 months up to 1.6 months).

Almost all the cytokines and time points displayed no significant difference between the pressure (canine-distal), tension (canine-mesial), and anchor (molar-mesial and molar-distal data pooled together) sites. Using the Tukey-Kramer HSD test, IFN-γ showed a significant difference at time point 9.5 months between the pressure and tension sites ($p=0.0385$). IL-12p70 also showed a significant difference at time point 5.4 months between the pressure and anchor sites ($p=0.0336$). TNF-α also showed a significant difference at time point 5.4 between the pressure and anchor sites ($p=0.0434$).

Qualitative changes in concentration of each time point compared to baseline are shown in Figures 4-8. There seems to be a lot of variation and both increases and decreases compared to baseline can be seen at each time point without a clear trend; if anything, it could be described as cyclic.

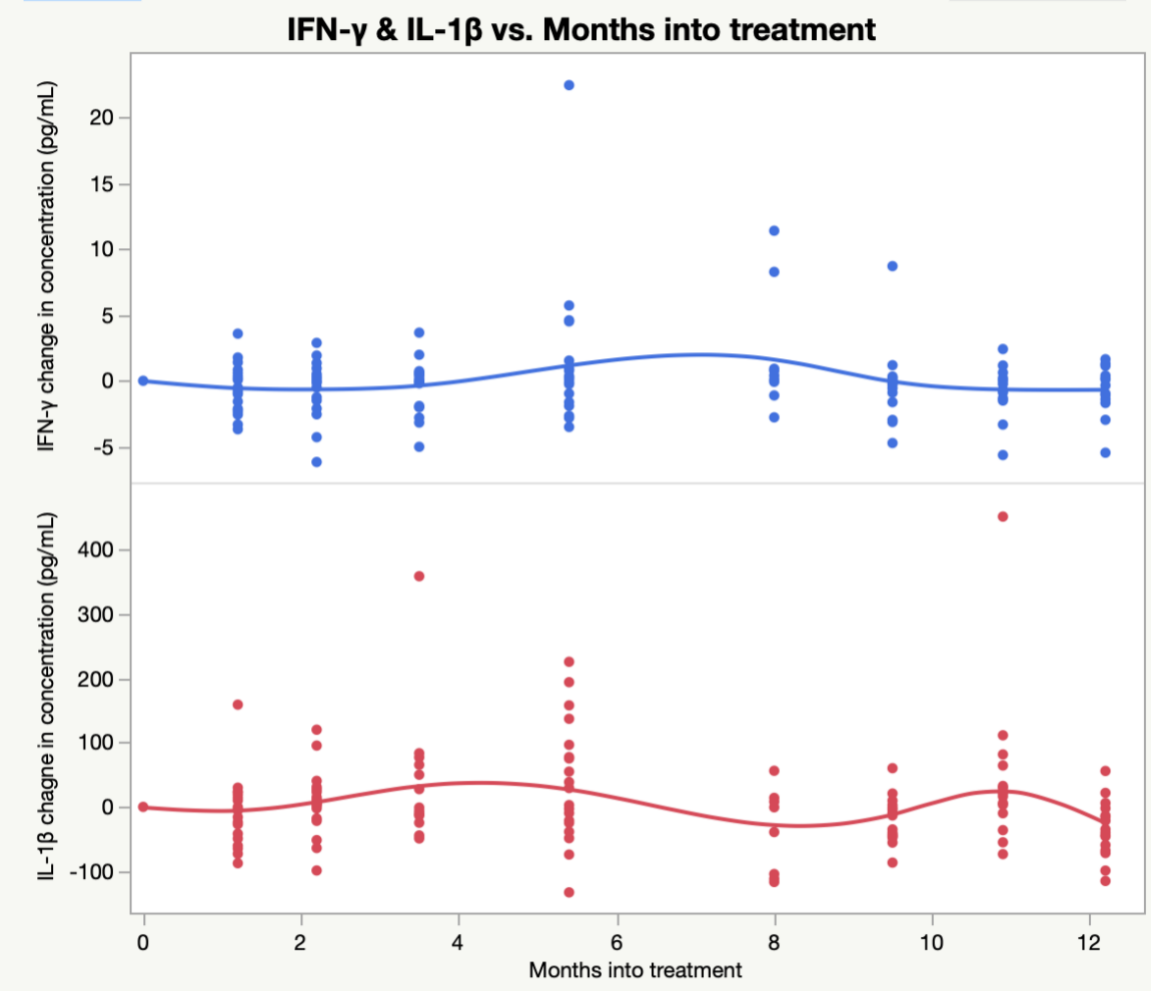


Figure 4. Change in concentration of IFN-γ and IL-1β over time.

Change in concentration compared to baseline for IFN-γ and IL-1β. A positive number means the concentration increased and a negative number means the concentration decreased.

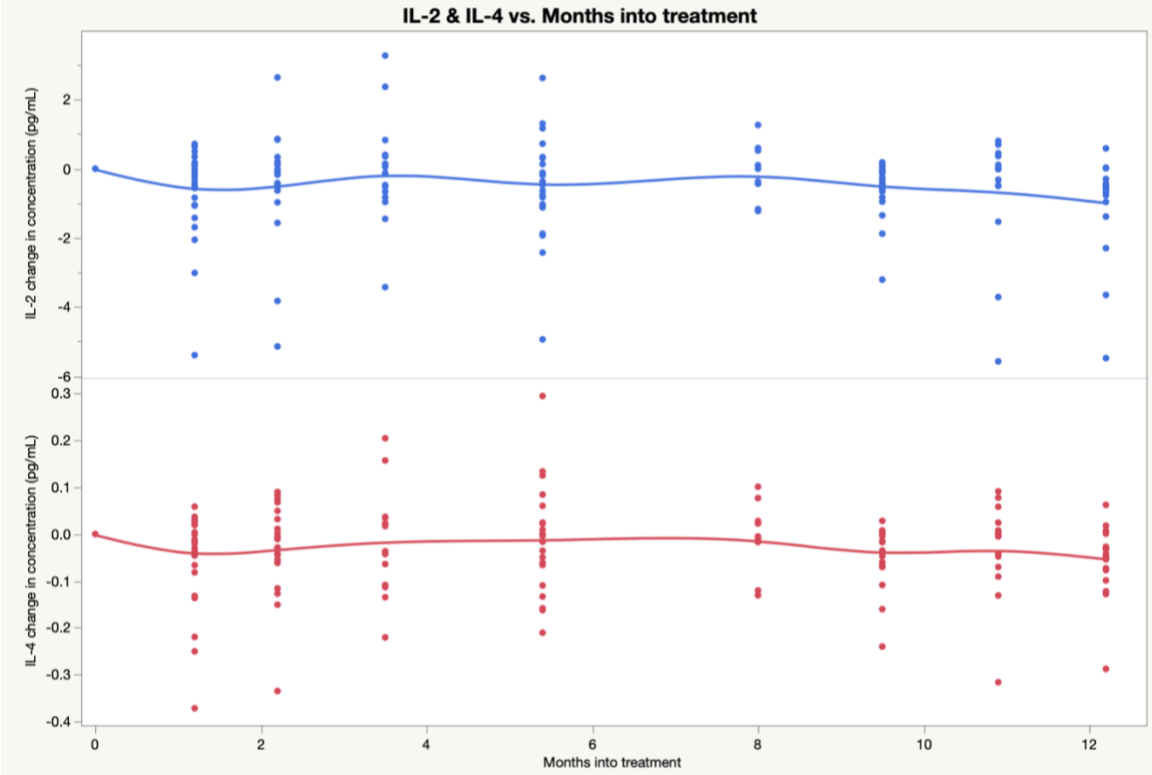


Figure 5. Change in concentration of IL-2 and IL-4 over time.

Change in concentration compared to baseline for IL-2 and IL-4. A positive number means the concentration increased and a negative number means the concentration decreased.

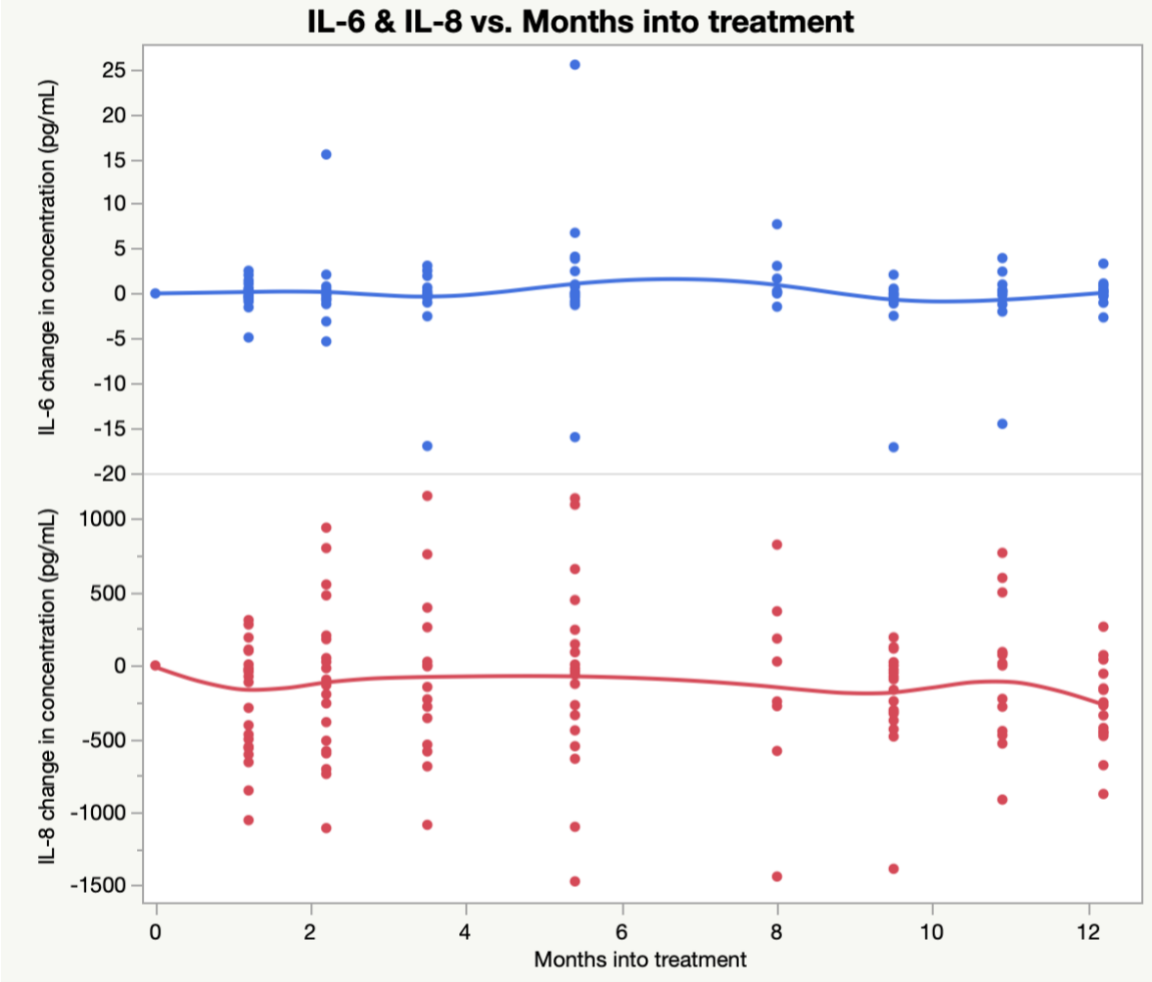


Figure 6. Change in concentration of IL-6 and IL-8 over time.

Change in concentration compared to baseline for IL-6 and IL-8. A positive number means the concentration increased and a negative number means the concentration decreased.

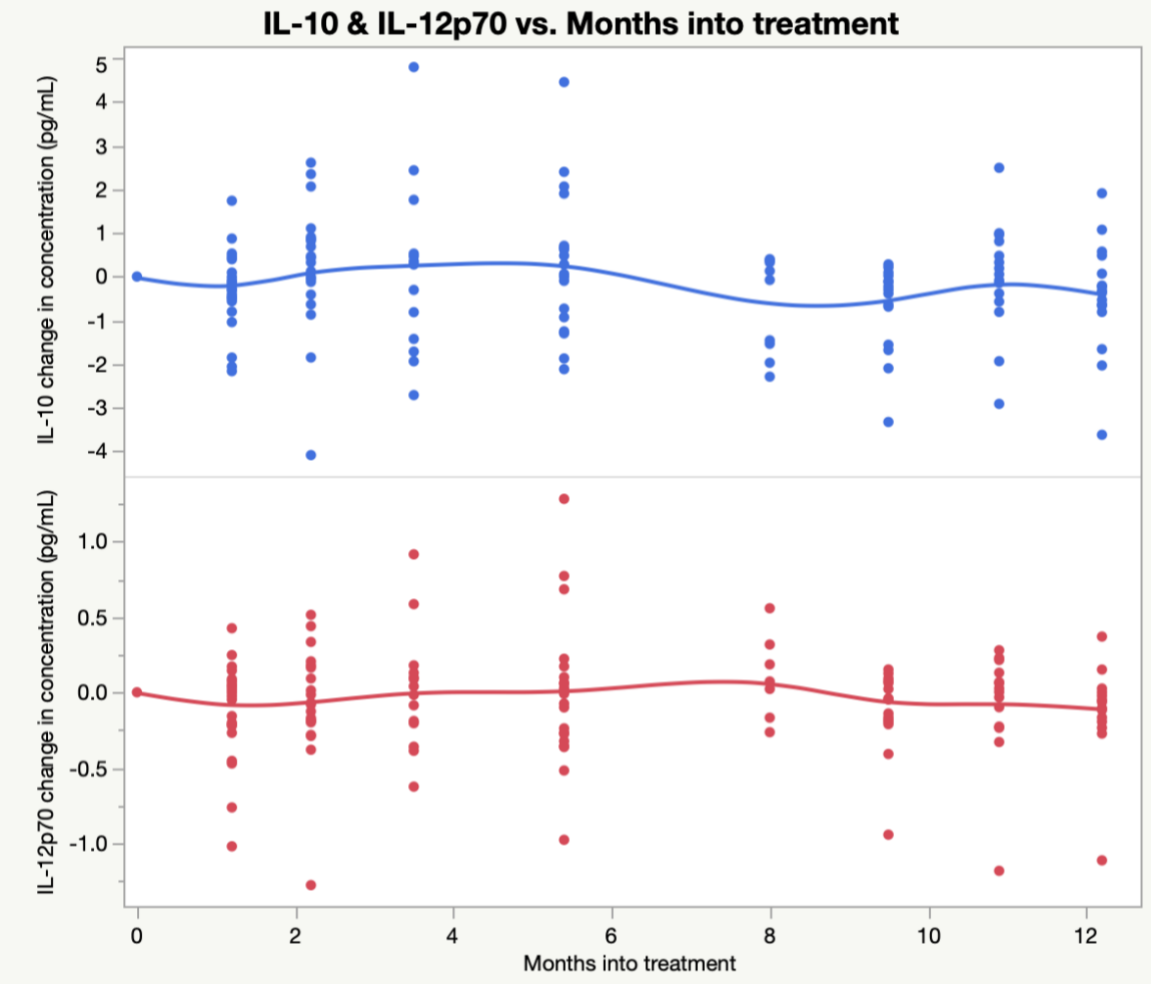


Figure 7. Change in concentration of IL-10 and IL-12p70 over time.

Change in concentration compared to baseline for IL-10 and IL-12p70. A positive number means the concentration increased and a negative number means the concentration decreased.

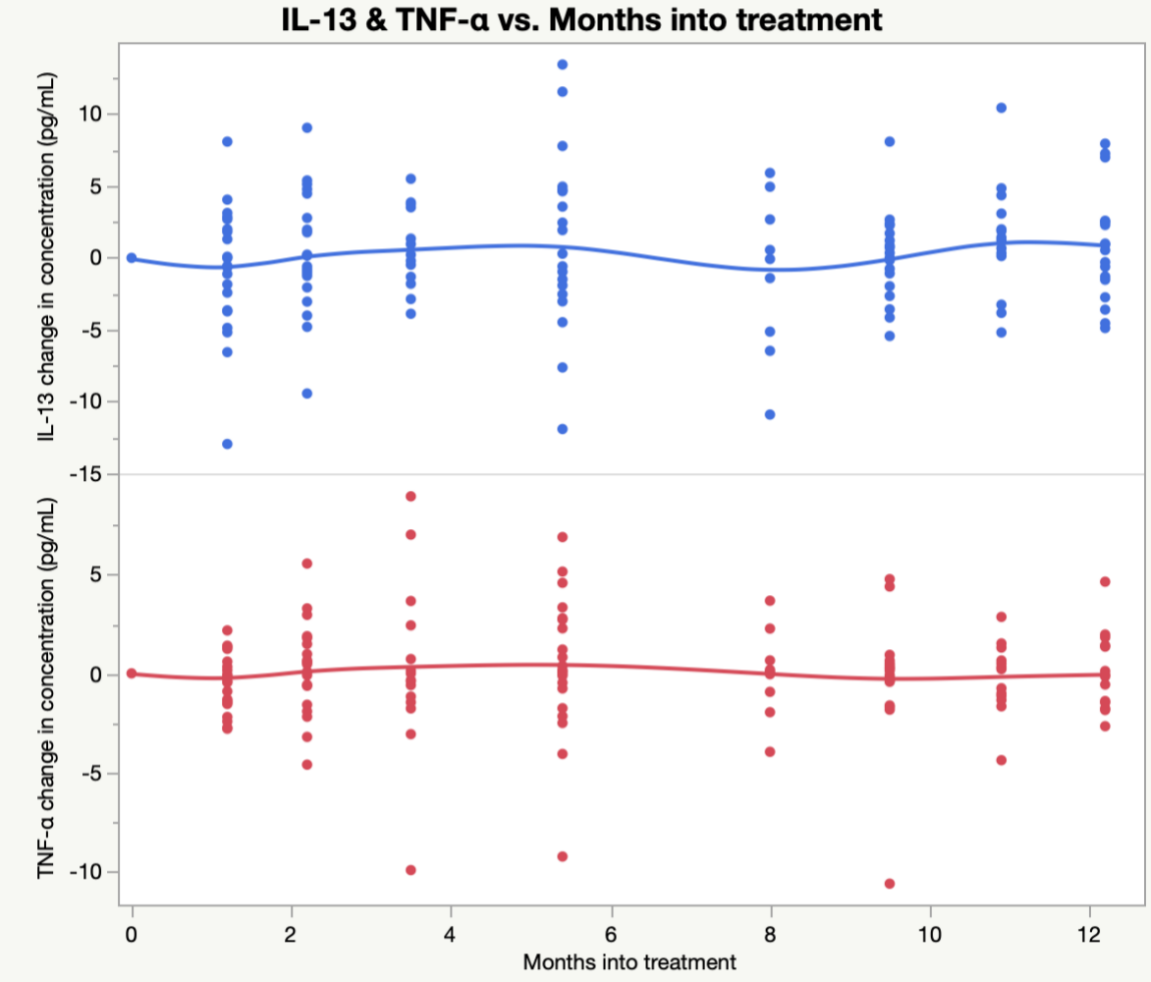


Figure 8. Change in concentration of IL-13 and TNF-α over time.

Change in concentration compared to baseline for IL-13 and TNF-α. A positive number means the concentration increased and a negative number means the concentration decreased.

# Chapter 5. Discussion

## 16S sequencing

There have been several studies that looked at the oral microbiome using 16S sequencing. Many of these studies looked at supragingival plaque or saliva samples. These studies and their findings are summarized in Table 3. Only one study[51] looked at subgingival plaque samples in 10 adult female patients treated with fixed appliances. The samples were taken from the maxillary and mandibular 1st molars and central incisors and pooled. The time points analyzed were 1) before treatment, 2) 1 month after placement of fixed appliances, and 3) 3 months after placement of fixed appliances. The same author also looked at subgingival samples in Invisalign patients in a separate paper.

Compared to their study, we did not pool our samples together, because we believed there may be differences between pressure vs. tension sites, and to evaluate this, we specifically looked only into maxillary 1st premolar extraction cases, where typically the canine will need to be retracted. Our subjects also included adolescent patients, since most orthodontic treatment is performed in individuals who are still growing. Hormonal changes during this time may also affect the composition of the subgingival biofilm, but the findings may be applicable to more orthodontic patients.

Table 3. 16S analysis of the oral microbiome.

| **Author and year of publication** | Zhao et al[52]<br>2020 | Guo et al[53]<br>2018 |
|---|---|---|
| **16S region and sequencing** | V4<br><br>Illumina HiSeq platform with a pair-end 250-bp strategy | V3-V4<br><br>Illumina MiSeq |
| **Appliance** | Invisalign | Invisalign |
| **Number of subjects** | 19 patients, unknown age and gender | 10 adult female patients |
| **Sample location** | Saliva | Pooled subgingival plaque from the maxillary and mandibular 1st molars and central incisors |
| **Time points** | 1. Before treatment (T0)<br>2. 3 months into treatment (T1)<br>3. 6 months into treatment (T2) | 1. Before treatment (T0)<br>2. 1 month into treatment (T1)<br>3. 3 months into treatment (T2) |
| **Findings** | • Phyla of Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Fusobacteira were predominant.<br>• Alpha diversity decreased at T1 and increased at T2.<br>• Beta diversity was different at T1 compared to T0 and T2.<br>• Relative abundance of Prevotella, Porphyromonas, and Peptostreptococcus decreased with treatment.<br>• Relative abundance of Capnocytophaga and Neisseria increased with treatment. | • No significant change in α diversity.<br>• Communities at T0 tended to cluster apart from T1 and T2 as shown by principal coordinates analysis.<br>• Relative abundance of the phylum Firmicutes and genus Mycoplasma was significantly increased at T0 compared with T2.<br>• No significant difference in the relative abundance of periodontal pathogens at the genus and species level.<br>• No significant difference in core microorganisms at the genus level. |

Table 3 continued

| **Author and year of publication** | Wang et al[54]<br>2019 | Guo et al[51]<br>2019 |
|---|---|---|
| **16S region and sequencing** | V3-V4<br><br>Illumina MiSeq<br><br>High throughput pyrosequencing | V3-V4<br><br>Illumina MiSeq |
| **Appliance** | Invisalign vs. fixed appliances | Fixed appliances |
| **Number of subjects** | 15 adult patients:<br>1. 5 fixed appliance patients<br>2. 5 Invisalign patients<br>3. 5 healthy controls | 10 adult female patients |
| **Sample location** | Saliva | Pooled subgingival plaque from the maxillary and mandibular 1st molars and central incisors |
| **Time points** | 6 months into treatment | 1. Before treatment (T0)<br>2. 1 month into treatment (T1)<br>3. 3 months into treatment (T2) |
| **Findings** | • The Invisalign group was not significantly different from the fixed appliance group<br>• The α diversity decreased in both treatment groups<br>• The richness and evenness of the oral microbiota were also disturbed in both treatment groups<br>• The predominant phyla were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, candidate division TM7, and Spirochaetes | • Alpha diversity was stable<br>• Beta diversity was higher at T2 compared to T0 and T1<br>• Relative abundance of core microbiomes at the genus level was relatively stable |

Table 3 continued

| **Author and year of publication** | Kado et al[55]<br>2020 | Koopman et al[56]<br>2015 |
|---|---|---|
| **16S region and sequencing** | V1-V2<br><br>Illumina MiSeq | V5-V7<br><br>454 FLX Titanium chemistry |
| **Appliance** | Fixed appliances | Fixed appliances |
| **Number of subjects** | 71 patients, including patients under the age of 18, all of Japanese ethnicity | 91 subjects, 10-16.8years of age<br><br><u>2 groups:</u><br>1. With a fluoride mouthwash<br>2. Without a fluoride mouthwash |
| **Sample location** | 1. Supragingival plaque from upper and lower anterior teeth<br>2. Unstimulated saliva | Supragingival plaque from the buccal surface of upper left premolars |
| **Time points** | <u>Supragingival plaque was collected:</u><br>1. Immediately before placement of fixed orthodontic appliances (T0)<br>2. 6 months into treatment (T1)<br><br><u>Saliva samples were collected:</u><br>1. T0<br>2. T1<br>3. Immediately after removal of appliances (T2), average of 40 months after placement | 1. 1 week before placement of fixed appliances (T0)<br>2. 6 weeks after placement (T1)<br>3. 12 weeks after placement (T2)<br>4. Debonding (TD)<br>5. 6 weeks after debonding (TD1)<br>6. 12 weeks after debonding (TD2) |
| **Findings** | <u>Supragingival plaque:</u><br>• Bacterial diversity at T1 was slightly more diverse than at T0<br>• There was increased abundance in supragingival plaque with time.<br>• Relative abundance of *Prevotella*, *Porphyromonas*, *Capnocytophaga*, *Parvimonas*, *and Selenomonas* spp were higher at T1 compared to T0<br>• Most of the bacteria which increased in abundance over time were obligate anaerobes<br>• All bacteria that decreased with time were aerobes or facultative anaerobes<br><br><u>Saliva:</u><br>• No differences in diversity between T0, T1, and T2<br>• Bacterial abundance increased over time<br>• Facultative or obligate anaerobes increased over time<br>• Aerobes or facultative anaerobes decreased with time | • No differences noted between the 2 groups (mouthwash vs. no mouthwash)<br>• No effect of time on the microbiome was found. There was no observable shift in the composition of the total community over time.<br>• Microbiome diversity became higher between T0 and T1 and lower between TD and TD1<br>• The phylum Actinobacteria decreased in abundance between T0 and T1 and increased at TD1 to TD2 compared to baseline<br>• The phylum Firmicutes increased in abundance at T1, TD, TD2 compared to baseline (T0).<br>• The abundance of Bacteroidetes decreased in TD1 and TD2 compared to baseline. |

**Cytokine analysis**

A lot of research has been completed regarding the cytokine profile in health and disease (gingivitis and periodontitis), and during orthodontic tooth movement. A systematic review by Kapoor et al[48] found 39 articles and concluded that there was an immediate release of inflammatory cytokines involved in bone resorption such as IL-1β and TNF-α. Ten studies found that peak levels of these cytokines occurred at 24 hours for continuous force application. Eight other studies reported a peak level anywhere between 4 hours to 6 months. Three studies found a decrease to baseline levels later on from 48 hours to 21 days. Two studies[57, 58] found that repeated activations when using interrupted force increases cytokine concentrations compared to continuous force, but the follow-up period was only 1-3 weeks. Significant heterogeneity was found among the studies, making it difficult to draw comparisons and conclusions.

A few longer-term studies warrant a more in-depth look. A study by Ren et al[21] (that was also included in the aforementioned systematic review) looked at concentration of IL-1β, TNF-α, IL-6, and IL-8 in GCF both short and long-term. 6 adult subjects participated in the short-term study. Separators were placed between the upper canines and upper first premolars for 24 hours. The maxillary 1$^{st}$ premolars were used as the experimental teeth and the antagonistic teeth were used as controls. Samples were collected from the distobuccal sites of the maxillary 1$^{st}$ premolars. 6 adolescent patients who needed extraction of maxillary 2$^{nd}$ premolars participated in the long-term study and 5 timepoints were examined: before tooth extraction and before orthodontic treatment (T0), after tooth extraction and initial alignment (T1); and 1, 2, and 3 months after

retraction of 1st premolars using light continuous force (T2, 3, 4). The authors did not describe how this force was applied.

For the short-term study, the authors found that TNF-α, IL-1β, and IL-6 levels were significantly increased at 24 hours, which was expected because they are part of the first wave of cytokines. For the long-term study, they did not find any statistically significant change in cytokine levels at all study points except IL-8 was elevated significantly at T1 and then decreased to baseline levels. IL-8 is a part of the second wave of cytokines. All cytokines tested returned to their baseline levels during retraction of the 1st premolars.

The authors concluded that when orthodontic force is applied, inflammatory cytokines are produced, triggering a cascade of cellular events, which then stabilizes over time until the next reactivation.

Başaran et al[25] (also included in the systematic review) looked at IL-2, IL-6, and IL-8 concentrations in GCF. They included 15 adolescent patients in their study who needed extractions of maxillary 1st premolars. They looked at 2 periods of orthodontic treatment: during leveling and during retraction using 150 g NiTi coil springs. GCF samples were collected from the mesial and distal sites of the maxillary canines. They were taken at baseline, 7 days, and 21 days into initial leveling of the teeth. Baseline samples for retraction were taken at 6 months, and then 7 and 21 days after starting retraction. They did not find any statistically significant differences except a decrease in the concentration of IL-8 on the 7th day of leveling.

Finally, Iwasaki et al[23, 59] (also included in Kapoor et al's systematic review) examined IL-1β levels in 7 patients undergoing maxillary 1st premolar extractions and distal movement of the maxillary canines over a period of 84 days. The mesial and distal site of the maxillary canine was used as the experimental sites, and a site at or near to a mandibular canine was used as the control site. Day 0 was defined as baseline, which was the day canine retraction forces were initiated. Time points were: Day 1, Day 3, and every 14 days (or as close as possible) until 84 days. All subjects received either a Nance or a Nance/TPA combination appliance. A calibrated spring was used for retraction. It appears that mandibular teeth were not bracketed during this time. They found that IL-1β levels increased within 3 days after loading, and then dropped to baseline by 14-28 days, and then continued to cycle about every 28 days, which the authors reported were similar to the results of Grieve et al[60] and Uematsu et al[61, 62] (all 3 articles were also included in Kapoor et al's systematic review).

Immediate and short-term changes were not examined in our study. Statistical analysis was only run comparing tension vs. pressure vs. anchor sites. Changes over time are shown qualitatively in graphs. Minimal difference was found between the 3 different sites. Several variables may explain the lack of significance. It appears that most studies found significant changes within 24 hours of force application, a time point not included in our study. Due to the number of different faculty and residents treating the patients, when and how force was applied to teeth greatly varied among the subjects. Many studies reported that cytokine levels returned to baseline levels over time, as the system stabilized, which could be another reason why we did not detect differences among the 3

sites. Our data also shows that cytokine levels appears to hover around baseline over long periods of time with periodic increases and decreases that do not show a clear linear pattern.

It does appear that our data for IL-1β displays some cycling as described by Iwasaki et al[23, 59], Grieve et al[60], and Uematsu et al[61, 62]. They reported a periodicity of 28 days, but in our data the increase and decrease in IL-1β concentration appears to range between 2-4 months.

The lack of a difference of cytokine levels among the pressure, tension, and anchor sites and insignificant change over longer follow-up periods found in our study and several others demonstrate the resilience of the oral cavity. It is encouraging to know that even under the burden of orthodontic tooth movement, when bone is under constant remodeling and there are unfamiliar appliances on the teeth and in the mouth, the oral cavity can adapt and stay healthy. Cytokine levels may be increased temporarily, but in the long-term over the course of treatment, they return back to baseline levels. This can also explain why orthodontic treatment doesn't appear to cause significant long-term periodontal damage (i.e. alveolar bone loss),[38] with many studies also finding improvement of inflammation and pseudopocketing after appliances are removed.[14, 49]

**Limitations**

There are several limitations to this study. The 2nd molar was sampled if it was erupted, otherwise the 1st molar was sampled. Most patients in the clinic do not have their 2nd molars bonded at the start of treatment, and none have the 2nd molars banded. Most of

the patients who participated in the study had their 1st molars banded because they had a Nance appliance. There was one patient who had a crown-design Herbst on the 1st molars. There have been studies showing that plaque and clinical periodontal parameters worsen when bands and brackets are placed on teeth, and that there are also differences between teeth that are banded vs. teeth that are bonded.[50] It was also extremely difficult to collect samples from the 2nd molar simply because it was so far back in the mouth. Grant et al[16] took samples from the distolingual of the 2nd molars, that may have been an easier site to sample from compared to the distobuccal since the cheek would not have been in the way.

The treatment progress varied greatly among patients due to patient compliance (e.g. late to appointments, missed appointments) and different philosophies of the faculty and resident providing treatment. Most patients had steady progress for space closure and canine retraction, but one still had almost the full amount of extraction space present after 1 year into treatment.

There was no control group of subjects who did not undergo orthodontics at all. A split mouth design would not have been ethical because it would have delayed placing braces on either the other quadrant or the lower arch for 1 year, which would likely increase treatment time by the same amount of time and complicate treatment mechanics. It would be difficult to recruit patients of the same age not undergoing orthodontic treatment to sample every 4-8 weeks but can be attempted in a future study. A control group would be beneficial to rule out the effects of hormonal changes during adolescence, as the microbiome can also be influenced by changes in hormone level.

The residents whose patients were participating in the study were asked to not have their patients brush at the beginning of the appointment until samples were collected, but occasionally the patients were still allowed to brush beforehand. During Covid-19, hydrogen peroxide mouth rinse was required at the beginning of all appointments. Most of the time samples were collected before the patient rinsed, but there were 1-2 appointments where the patient rinsed before samples could be collected.

A larger sample size would be beneficial as only 6 patients had the full year of follow-up completed, 2 were followed for 10 months, and 1 was followed for 8 months.

One patient had a change in treatment plan mid-treatment and upper 2$^{nd}$ premolars were extracted instead of upper 1$^{st}$ premolars.

Reliability testing would have improved the quality of the study if tooth movement had been measured on models using digital calipers as done in several other studies. This would have added more time for the patient during the sampling process, which may not have been feasible due to interference with the clinic and resident's schedules. More frequent calibration of the researchers completing sample collection and clinical measurements or limitation of sample collection/clinical measurements to 1 researcher would have also been.

Finally, the clinic was closed for 3 months from March 2020 to June 2020, during which no samples were collected and no patients were seen for adjustments. This caused a gap in sample collection, which occurred during months 4-9.

**Future directions**

Completing the analysis on the 16S data would be our first goal for the immediate future. Large data such as longitudinal sequencing data can be challenging to break down and understand. There are new statistical methods being developed such as mixOmics and longitudinal analyses in Qiime that we will be using to analyze the data and even integrate the cytokine data with the 16S sequencing data.

Controlling variables such as different treatment protocols, mechanics, and appliances would be beneficial in studying tension vs. pressure sites. Applying the same force at the same time such as in Grant et al's study may have given clearer results. The patient treated with a Herbst had no force applied to the canine; the tooth was allowed to drift distally until the space was almost closed. Two patients were treated with rapid palatal expanders at the beginning of treatment (no samples were taken until after expansion was completed). The rest had a Nance in for anchorage, except one patient who did not have appliances, and was treated with brackets and elastics. It would have been very difficult to standardize the treatment mechanics, time points, and appliances in this study because of how many different providers treated the patients (5 residents supervised by 5 faculty) and limitations of everyone's schedules.

Increasing the sample size would also be beneficial, as well as having a control group to rule out the effects of hormonal changes on the subgingival plaque. Reliability testing of investigators taking the samples and measurements would also help in reducing heterogeneity of the results.

Because cytokines occur in cascades, it may be helpful to look whether an increase in one cytokine leads to a different cytokine increasing in concentration, and whether these spikes are caused by an activation in force. Adding some earlier timepoints, such as 1 hour, 4 hours, 24 hours after application of force will further help delineate the cytokine response.

**Conclusions**

The levels of pro-inflammatory cytokines in the gingival crevicular fluid such as IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α appear to fluctuate around baseline over long periods of time, at times increasing and at times decreasing.

No difference was found among the pressure, tension, and anchor sites in terms of cytokine concentration at any time point for all cytokines examined except for IFN-γ at 9.5 months (decrease in concentration in pressure sites from baseline was significantly greater compared to tension sites), IL-12p70 at 5.4 months (decrease in concentration from baseline in pressure sites was significantly greater compared to anchor sites), and TNF-α at time point 5.4 months (decrease in concentration from baseline in pressure sites was significantly greater compared to anchor sites).

These findings suggest that the oral cavity is resilient and can adapt to the burden brought upon by orthodontic forces without significant detrimental or permanent effects.

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