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Levels of Interleukin-1β and Interleukin-17 in the Tension and Compression Sites of Teeth during Orthodontic Movement

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LEVELS OF INTERLEUKIN-1β AND INTERLEUKIN-17 IN THE TENSION AND

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by

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Abstract

Orthodontic tooth movement requires coordinated bone resorption and deposition in response to mechanical stimuli. Underlying this process is a complex network of biological signaling to activate osteoclasts and osteoblasts. The broad category of small proteins known as cytokines include many potential mediators involved in complex interactions to initiate tooth movement. One of the signaling factors is interleukin-1β (IL-1β), which plays a critical role in inflammation and bone erosion via the activation of osteoclasts. IL-1β is a key cytokine involved in osteoclast differentiation, multinucleation, and overall survival. Recently, interleukin-17 (IL-17) has been identified as a potent inducer of inflammation that influences bone homeostasis. IL-17 affects a broad range of cell types and enhances the production of other pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α. To date, few studies have examined the role of IL-17 in orthodontic tooth movement. However, studies focusing on the role of IL-17 in bone homeostasis suggest that IL-17 may be a key mediator of bone resorption during orthodontic tooth movement. The present study expanded upon previous research by Faulkner (2011) which demonstrated elevated IL-6 levels in GCF within 24 hours of orthodontic force activation. The aim of this study was to evaluate whether increased levels of bone resorptive cytokines (IL-17 and IL-1β) can be detected in GCF from the compression (resorptive) sites after application of orthodontic force. This study also evaluated the expression of IL-17 and IL-1β at various time points to determine if there was a temporal relationship between the expressions of these two cytokines. GCF samples were collected 1, 6, and 24 hours after initial activation from mesiobuccal (MB) and distolingual (DL) sites of experimental and control teeth from 9 patients undergoing orthodontic treatment. Control teeth were not bonded, thus no force was applied. Patients returned in 5-7 weeks for their 1st retie activation visit. GCF

samples were collected again at the same time intervals. GCF was sampled three times for each site and time point and pooled for volume analysis. A Bradford assay was performed to obtain total protein levels of each pooled sample at each time point. MILLIPLEX® MAP (multi-analyte profiling) assays were used to detect IL-1β and IL-17 in the GCF samples from both the tension and compression sites. IL-1β and IL-17 levels for both experimental and control sites were compared with a paired t-test. Levels of IL-1β at the mesiobuccal sites peaked at 6 hours after orthodontic force application at both the initial and retie visits. While all previous studies reported peak expression of IL-1β at 24 hours, this present study was the only study to measure cytokine levels at 6 hours post force activation. Levels of IL-1β peaking at 6 hours may imply that the inflammatory response is most reactive at 6 hours post force application. Levels of IL-17 peaked at the mesiobuccal site 24 hours after force application at both the initial and retie visits. There seems to be no correlation between the temporal expression of IL-1β and IL-17. It is possible that better detection methods or additional patients and sites may help to determine smaller variations in IL-17 at compression and tension sites. This may also help with defining a temporal relationship between the expressions of IL-1β and IL-17.

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Chapter 1

Introduction

The skeleton is a rigid yet dynamic organ that undergoes continuous remodeling activity throughout life. Bone remodeling involves removal of mature or damaged bone by osteoclasts and deposition of new bone by osteoblasts. This turnover process is essential for the maintenance, size, and quality of the skeleton (Hadjidakis and Androulakis, 2006). In childhood, bone remodeling allows for the formation and growth of new bone, and for bones to grow in size and change in shape. In adulthood, remodeling repairs damaged or fractured bone to maintain a healthy skeleton.

In the field of orthodontics, clinicians use continuous gentle pressure from archwires to move teeth within alveolar bone to improve dental alignment and correct malocclusions. This is made possible by bone remodeling around the tooth. Bone remodeling in the skeleton is controlled locally and systemically by cytokines, hormones, and other proteins. Bone remodeling of teeth in the oral cavity depends on a tightly regulated cascade of signals expressed by cells within the periodontium. Alveolar bone and tissue remodeling in response to an applied force is initiated by a complex coordination of biochemical events, thus an understanding of its processes is of great interest to orthodontists.

During orthodontic tooth movement, the equilibrium between bone apposition and bone resorption is disrupted. Mechanical strain from orthodontic forces causes minor reversible injury to the alveolar bone, periodontal ligament (PDL) and surrounding periodontium. These forces alter blood flow and change the localized electrochemical environment within the periodontal space (d'Apuzzo *et al.*, 2013). At the compression site, in the direction where force is applied, constriction of the periodontal ligament microvasculature releases various chemo-attractants that

recruit relevant inflammatory modulators to the injured site (Lindskog and Lilja 1983). Osteoclasts, the cells responsible for bone resorption, are recruited along with other phagocytic cells to dissolve the necrotic periodontal ligament and underlying bone and cementum. Osteoclasts are giant multinucleated cells that are derived from hematopoietic cells from the bone marrow (Gothlin and Gustav, 1976). The folding of their plasma membrane facing the bone matrix is called the ruffled border, and it secretes lysosomal enzymes such as tartrate resistant acid phosphatase and cathespin K to dissolve and resorb the underlying organic bone matrix, mainly type 1 collagen (Blair, 1998). As a result, they form Howship's lacunae which are small depressions in the bone (Gothlin and Gustav, 1976). Until osteoclasts remove the necrotic bone, tooth movement is impeded.

Once necrotic bone is removed and the tooth is allowed to move in the direction of the force applied, bone forming cells, osteoblasts, are recruited to the tension site and are responsible for the production of bone matrix (King et al, 1991). Osteoblasts are found along the surface of the bone and line the newly formed or growing layer of bone matrix. Originating from multipotent mesenchymal stem cells, osteoblasts produce osteoid, the non-mineralized organic component of bone consisting primarily of collagen (Fornasier, 1977). Mineralization soon begins after collagen deposition and slowly increases until the osteoid is fully mineralized into mature bone (Dimitrios and Androulakis, 2006).

While there are multiple biochemical cascades that regulate bone homeostasis, one of the most predominate pathways involve the interleukin 1 (IL-1) cytokine family as signal transducers. One of the signaling factors is IL-1β, which induces bone erosion by activating osteoclasts (Davidovitch *et al*., 1988). In addition to activating osteoclasts, IL-1β is also a key cytokine involved in osteoclast differentiation, multinucleation, and overall survival

(Davidovitch *et al*., 1988). IL-1β has the potential to drive osteoclast differentiation via a Receptor Activator of NF-kappaB Ligand (RANKL)/RANK-independent mechanism in micro-environmental conditions (Suda *et al*., 1999, Boyle *et al*., 2003). Although there are many studies on IL-1β and its receptors, relatively less research has focused on its role during orthodontic tooth movement. In addition, while IL-1β in gingival crevicular fluid (GCF) has been measured during tooth movement at 1 hour and 24 hours after force activation (Dudic *et al*., 2006), there are no studies describing the expression between 1 and 24 hours.

Interleukin17 (IL-17) is a T cell-derived cytokine that plays a role in osteoclast development. IL-17 has been recently identified and its mechanism is not fully understood. Research has shown that IL-17 is expressed in the early stages of immune response and acts on osteoblasts resulting in the expression of osteoclast differentiation factor, also known as RANKL, which induces osteoclast progenitors to differentiate into mature osteoclasts (Kotake *et al.,* 1999). However, little is known about the role that IL-17 plays in orthodontic tooth movement.

Previously, a study has been conducted to evaluate levels of IL-6 in tension vs compression sites during orthodontic tooth movement (Faulkner *et al*., 2011). Cytokine levels in GCF were examined at 0 hours, 1 hour, 6 hours and 24 hours after orthodontic force activation. Results showed that IL-6 levels in GCF peaked at 6 hours rather than the previously accepted peak at 24 hours. No study has been conducted to test levels of IL-1β at 6 hours after force activation. Other studies have only evaluated its presence following 1 and 24 hours after force application. Since IL-1β has a direct role in the chemical cascade that initiates osteoclast production, it is of great interest to examine a more detailed timeline of IL-1β cytokine expression.

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This present study will be the first attempt at characterizing the early expression of IL-1β and IL-17 immediately following force activation during orthodontic tooth movement. Comparing the levels in the compression and tension sites within 24 hours of force application will provide data on the temporal initiation of cytokine expression as well as the timing of peak expression during the initial stages of orthodontic tooth movement. Not only is it important to study these cytokines as biomarkers in the biological process of tooth movement, but it is also important to study the temporal expression of these cytokines if attempts are to be made to maximize the rate of tooth movement, decrease overall treatment time, manage root resorption and determine the ideal interval for retie appointments. Understanding the temporal expression of these cytokines may also help clinicians better treat patients prone to root resorption. By establishing a temporal relationship between orthodontic tooth movement and levels of these cytokines, it could help clinicians establish optimal retie intervals and take advantage of these cytokine responses to most efficiently treat their patients.

Research Questions & Hypotheses

1. Will there be increased levels of bone resorptive cytokines (IL-1β and IL-17) detected in GCF from the compression (resorptive) sites after application of orthodontic force?

> H_0: Increased levels of bone resorptive cytokines will be not be detected in GCF from the compression sites after application of orthodontic force.
>
> H_A: Increased levels of bone resorptive cytokines will be detected in GCF from the compression sites after application of orthodontic force.

2. Will the largest increase (peak) in the level of bone resorptive cytokines (IL-1β and IL-17) be detected within 24 hours (1 or 6 hr time points) after orthodontic force application?

H_0: The largest increase in the level of bone resorptive cytokines will be detected at 24 hours or more after orthodontic force application.

H_A: The largest increase in the level of bone resorptive cytokines will be detected within 24 hours after orthodontic force application.

Chapter II
Literature Review

Orthodontic tooth movement occurs when a force is placed on a tooth causing compressive force against the PDL and bone. As a response to this constant mechanical force, the PDL and bone remodel, which allows the tooth to move. Bone resorbs in the direction of force applied and bone deposits on the side from where the tooth has been displaced. This combination of movement allows teeth to change position within alveolar bone. Thus, in orthodontic tooth movement, there are two distinct opposite sides that are referred to as the tension site, where displacement of tooth causes a force to the PDL causing bone formation, and a compression site, where compressive forces are generated by the root moving against bone causing the bone to resorb (Sandstedt, 1904).

Phases of tooth Movement

In essence, tooth movement can be divided into three stages. Burstone (1962) outlined tooth movement in 3 phases: an initial phase, a lag phase, and a post lag phase.

In the initial phase, there is immediate tooth movement almost immediately after force application. This is due to relatively unhindered movement within the PDL space. After the initial phase, there is a lag period with little to no displacement of the tooth. This lag phase is associated with hyalinization in compressed regions of the PDL (Dalstra *et al.*, 2006). Hyalinization is the sterile necrosis of tissues that occurs in local pressure zones. Although orthodontists try to use gentle light forces, hyalinization is almost unavoidable. The alveolar bone surface is rough and jagged, thus even light forces may produce high enough stress to cause hyalinization (Dalstra *et al.*, 2006). These hyalinized areas cannot generate and signal progenitor cells to differentiate into osteoclasts. Osteoclasts are needed from undamaged adjacent marrow

cells to remove the necrotic tissue: This is referred to as undermining resorption (von Bohl and Kuijpers-Jagtman, 2008). No tooth movement occurs until all necrotic hyalinized tissues have been removed. After this lag phase comes the post lag phase where the rate of movement is suddenly increased and tapers to become a steady gradual movement (Burstone, 1962).

An additional study completed by Pilon et al. (1996) further defined phases of tooth movement. They categorized tooth movement into four phases with distinct time points. In the study, phase 1 lasted 3 - 4 days or less for 75% of the cases. The first phase accounts for the initial tooth movement within the PDL socket. The second phase, also known as the arrest phase, is associated with little to no tooth movement due to hyalinization of the PDL. Phase two usually lasts 4-20 days after force activation. Phase 3 is characterized by an accelerated rate of tooth movement, suggesting that remodeling in this stage is at its peak capacity. Phase 4 is characterized by a linear rate of tooth movement. In Phase 4, the tension side shows osteoblastic proliferation and activity. Pericytes, which are fibroblast like cells that line the PDL capillaries, recruit osteoblast progenitor cells to start producing new bone matrix. Tooth movement during phases 3 and 4 are continuous and not interrupted.

Theories of Orthodontic Tooth Movement

Historically, there have been many theories describing the mechanisms of orthodontic tooth movement. The following reviews the most widely accepted theories.

Pressure Tension Theory

The pressure and tension theory is a culmination of research published by Sandstedt (1904), Oppenheim (1911), and Schwartz (1932). It states that when a force is placed on a tooth, the tooth reacts and moves within the periodontal space due to cellular changes produced by chemical messengers in the periodontium. It then generates a "pressure side" and a "tension

side". Force on the pressure side causes alteration in blow flow associated with compression within the PDL and causes cell death (Schwartz, 1932). Vasodilation leads to the formation and release of chemical messengers which activates specific cells for orthodontic tooth movement. When the PDL is mechanically stimulated osteoclasts appear within 48 hours. The first-wave arrives from a local cell population within the PDL. The second wave is brought from distant areas via chemical signaling. Osteoclasts proceed to remove the dead bone cells which lead to tooth movement. On the tension side, the PDL fiber bundles are stretched to stimulate blood flow and promote osteoblastic activity and osteoid deposition. This heightened osteoblastic activity results in fiber production and deposition of newly formed bone.

Bone Bending Theory

Farrar (1888) theorized that alveolar bone bending leads to orthodontic tooth movement. Further research from Baumrind (1969) confirmed his hypothesis. Baumrind stated that the PDL is a continuous hydrostatic system that diffuses forces evenly so there is no distinct pressure or tension side. His hypothesis, called the bone bending theory, states that orthodontic forces are transmitted to all tissues near its application and bends the bone, tooth and surrounding periodontium. Bone is elastic in nature and bends readily in response to force. When bone is bent and held in the new deformed position, active biologic processes begin the bone turnover process. These biologic signals originate from agitated cells lying perpendicular to stress lines created by the bone bends. As bone modifies and restructures to accommodate forces acting on it, the tooth moves and forces dissipate. The bone bending cycle starts all over when new force is applied, such as during the patient's retie appointments where stiffer and larger wires are placed. Grimm (1970) discovered that alveolar bone bending accounts for 0.6-25% of total

tooth movement. This proved that mechanical strain in bone is one of the mediating factors which convert an extrinsic force to cellular bone response.

Bioelectric Signals

The bioelectric signals theory proposes that exogenous forces create electric potentials in stressed tissue. These electric potential charges cause macromolecules to bind to or move across cell membranes to induce bone turnover (Bassett and Becker, 1962). Previous studies on the skeleton showed that osteogenesis can be triggered by placing appropriate currents on long bones (Friedenberg *et al*., 1970). To apply this theory to orthodontic tooth movement, Zengo *et al*. (1974) measured stressed induced electric potentials from the dentoalveolar complex during application of orthodontic force in beagles. The study resulted in the discovery that the concave surfaces of stressed bone is charged with electronegative potentials which showed elevated osteoblastic activity and the electropositive signals from convex surfaces showed greater osteoclastic activity. The study linked bioelectric responses from bone bending, both piezoelectric and streaming potentials, to orthodontic tooth movement.

From each theory's initial proposal to now, ongoing studies have not proved nor disproved one hypothesis to be the evident truth. However, the pressure and tension theory is most widely accepted. Recent studies have suggested that both physical and biologic processes are needed for tooth movement, with force application, tissue cells, alveolar bone, and PDL responding cohesively to initiate bone remodeling.

Cytokines

Cytokines are soluble secreted proteins released by cells and are primarily involved in cellular interaction and communication (Zhang and An, 2009). Cytokines have the ability to act on cells that secrete them, on a cell close by or far away, and some are pro-inflammatory while

others are anti-inflammatory. These proteins function as intercellular messengers and regulate cellular activity throughout the body. Cytokines are pleiotropic, meaning a single cytokine can cause different effects depending on the cell types they bind to or interact with. One cytokine can stimulate varying effects on target cells depending on the microenvironment (Simpson *et al.*, 1997). Certain cytokines can also influence similar biological pathways and may even have common receptors (Simpson *et al.*, 1997).

Cytokines are needed to initiate bone turnover during orthodontic tooth movement. The transduction of forces on PDL and bone triggers a cellular biological response mediated by inflammatory cytokines that are responsible for tissue and bone remodeling. Orthodontic forces result in an increase of PDL neurotransmitters such as substance P, which causes vasodilation and production of inflammatory cells and cytokines (Norvall *et al*., 1998). Once they arrive to the local injury site, cytokines and inflammatory mediators act as signaling proteins to directly influence bone remodeling (Davidovitch *et al*., 1988). Some important cytokines that are involved in bone metabolism include RANK, RANKL, OPG, bone morphogenetic proteins (BMPs), interleukin 1 family, interleukin 6, interleukin 17, and TNF-α in addition to many others (Krishnan & Davidovitch, 2009).

RANK/RANKL/OPG

Understanding the relationship between RANK, its ligand (RANKL), and osteoprotegerin (OPG) was a pivotal discovery that clarified the roles of osteoblasts and stromal cells in bone homeostasis. This pathway has been crucial in understanding osteoclast formation and activation. RANK is a type I homotrimeric transmembrane protein and is expressed in a variety of cells (Boyce and Xing, 2007). RANKL is a type II homotrimeric transmembrane protein (Ikeda *et.al*, 2001) that is derived from the TNF family and is essential for bone metabolism.   RANKL

regulates the development, maintenance and activation of osteoclasts. It is expressed by osteoblasts and stimulates local osteoclasts to resorb bone which in turn stimulates local osteoblasts to synthesize bone. This feedback process called "coupling", helps maintain harmony and balance (Udagawa *et al*., 2000). Most factors that stimulate osteoclast formation also stimulate RANKL expression in osteoblasts. RANKL is also highly expressed in immune regulating tissues such as lymph nodes and thymus. OPG is a homodimer protein that serves to protect against bone loss and is expressed in osteoblasts, bone marrow, spleen and heart tissues (Wada *et al*., 2006). It is secreted by osteoblasts and osteogenic stem cells to upregulate bone density (Simonet *et al*., 1997).

Osteoblasts produce both RANKL, which initiates bone resorption, and OPG, which inhibits bone resorption. (Kearns *et al.*, 2008; Khosla, 2001). When RANK binds to RANKL osteoclastogenesis is initiated and osteoclasts are activated. However OPG serves as a soluble decoy receptor to RANKL (Simonet *et al,* 1997). Both OPG and RANK show affinity to the RANKL, thus OPG competes with RANK to bind to RANKL. When OPG binds to RANKL, it prevents the cell to cell signaling of RANKL on osteoclast precursors resulting in inhibition of osteoclastogenesis (Yasuda *et al*, 1998). The OPG–RANKL complex counterpoises the effect of the RANK–RANKL complex, thus playing an important role in bone homeostasis and metabolism. If OPG is not produced, RANKL exclusively and freely binds to RANK and begins the osteoclastogenesis pathway. This is further proven in knock-out studies of OPG in mice which lead to severe osteoporosis with spontaneous fractures (Yasuda *et al.,* 1998). RANKL has a different effect on osteoclastogenesis depending whether it binds to RANK or OPG. This signaling pathway is essential for bone homeostasis and a disruption of either cell can lead to metabolic bone diseases (Kohli, Kohli 2011).

Biomarkers

A biomarker is substance that is measured and used as an indicator of biologic processes and pathological processes (Strimbu and Tavel, 2010). The United Nations and International Labor Organization defined a biomarker as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence or outcome of disease" (WHO). A biomarker should be specific and sensitive so it can provide information about the changing biological conditions. IL-1β is a proven biomarker for cellular changes during orthodontic tooth movement (Kumar *et al.,* 2015). Many studies have found elevated levels of IL-1β in GCF from experimental teeth after force is applied (Grieve *et al*, 1994, Uematsu *et al*, 1996, Salla *et al*, 2012). In summation, their studies concluded that the increase of IL-1β is associated with bone remodeling during orthodontic tooth movement. IL-17 has been studied less extensively. Although there are studies quantifying levels of IL-17 in patients with root resorption associated with orthodontic treatment, and in patients with periodontitis, no study has evaluated if IL-17 is associated with normal orthodontic tooth movement and bone remodeling. Further knowledge of these biomarkers gained from this present study may be used to identify changes in the periodontal microenvironment and may be clinically useful to help improve and shorten orthodontic treatment.

IL-Iβ

There are 11 members of the Interleukin 1 (IL-1) family of ligands. IL-1β is part of the IL-1 cytokine family and is critical to immune response and function. IL-1β in particular has been extensively studied due to its control over inflammation at the receptor and nuclear levels. (Weber *et al*., 2010).

IL-1β is released from a wide variety of cells including keratinocytes, fibroblasts, synoviocytes, endothelial, neuronal and immune macrophage and mast cells (Ren and Torres, 2009). IL-1β can induce gene expression and synthesis of many proteins that cause fever, lowered pain threshold, vasodilatation, and hypotension including cyclooxygenase type 2 (COX-2), type 2 phospholipase A, and prostaglandin-E2 (PGE2). This also affects immune responses as PGE2 is a common mechanism for nonspecific suppression of T cell responses (Ren and Torres, 2009). In addition to inducing the production of certain chemokines, IL-1β also increases the expression of adhesion molecules and promotes the infiltration of inflammatory and immunocompetent cells from circulation into extravascular spaces to increase inflammation in tissues (Dinarello, 2009).

In the periodontium, IL-1β is released from fibroblasts, macrophages, cementoblasts, osteoblasts and osteoclasts (Alhashimi *et al*., 2001) in response to mechanical stress. At the biochemical level, orthodontic forces stimulate the release of prostaglandins, growth factors and cytokines, including IL-1β. Studies have shown that an increase in IL-1β correlates with the increase in osteoclast numbers (Alhashimi *et al*., 2001), suggesting that this cytokine is an important initiator of osteoclastogenesis. During the initial stages of tooth movement, IL-1β is secreted by osteoclasts as a response to mechanical force. In the later stages, IL-1β is secreted by macrophages that have accumulated at compressed areas (Davidovitch *et al*., 1988). IL-1β recruits other inflammatory cells to the damaged tissue site and binds to its receptor on the surface of pro-osteoblastic cells and can activate both RANKL and OPG genes. The ubiquitous nature of IL-1β combined with the increased expression in both RANK and RANKL are needed to remodel the periodontium and resolve tissue stress from orthodontic forces.

To further demonstrate IL-1β's role in orthodontic tooth movement, studies using exogenous injections of interleukin-1 receptor antagonist (IL-1Ra) of IL-1β have been conducted. Salla *et al.* (2012) injected IL-1Ra, a naturally occurring IL-1β antagonist, in mice to determine its effects on tooth movement. They discovered that mice treated with IL-1Ra showed diminished orthodontic tooth movement and a decreased number of TRAP positive osteoclasts compared to the untreated group. In the treated groups, ELISA analysis revealed lower levels of IL-1β and TNF-α. Iwasaki *et al*. (2006) also examined the relationship between levels of IL-1β and velocity of tooth movement. It was concluded that elevated levels of IL-1β are associated with faster tooth movement. Iwasaki *et al.* conducted an additional study in 2009 to further investigate the biologic factors that affected tooth movement, particularly IL-1β and IL-1Ra. They analyzed GCF samples on canines that were distally translated under varying force levels. Their conclusion supports other studies fortifying the idea that increased levels of IL-1β and decreased levels of IL-1Ra are associated with accelerated tooth movement.

With applied mechanical forces, fibroblasts, macrophages and cementoblasts produce IL-1β which diffuses into the gingival crevicular fluid. Studies have been conducted to evaluate the concentrations of IL-1β during orthodontic tooth movements at 0, 1, 24, and 48 hours after force application (Grieve *et al*, 1994). Concentrations of IL-1β peaked at 1 and 24 hours with 24 hours being the highest (Grieve *et al*, 1994). However, the peak elevation or significant changes within 24 hours is unknown. Our study aims to verify these established conclusions and also add to the timeline of IL-1β expression by evaluating levels at 0, 1, 6 and 24 hours after force activation.

IL-17

IL-17 is an inflammatory cytokine that is produced by activated T-helper 17 cells (Th17) from T-helper 1 precursors (Yao *et al*., 1995). Studies have shown that IL-17 acts as a crucial mediator of many autoimmune diseases such as multiple sclerosis, asthma and rheumatoid arthritis (Agache *et al*., 2010, Al-Saadany *et al*., 2016). IL-17 has been shown to stimulate the expression of RANKL in synovial cells in rheumatoid arthritis. Functionally, IL-17 mediates pro-inflammatory responses in host defense and inflammatory diseases. IL-17 promotes the production of cytokines and chemokines to attract neutrophils and macrophages to inflammation sites. IL-17 receptors are commonly expressed on fibroblasts and epithelial cells, and among innate immune cells, such as macrophages and neutrophils (Jin and Dong 2013).

Due to its extensive involvement in the inflammatory pathway especially with rheumatoid arthritis, most studies have examined the role of IL-17 in pathologic skeletal remodeling. Until recently, IL-17's role in bone remodeling was not clear. But recent discoveries have identified that IL-17 stimulates the production of MCSF and RANKL in osteoblast and mesenchymal stem cells (Lee, 2013). This action leads to a greater production of osteoclasts to shift the balance of bone homeostasis to bone resorption. When IL-17 is in the presence of inflammatory cytokines, the combination induces the differentiation and activation of osteoclasts (Sato *et al.*, 2006). In addition, specifically in the presence of TNF-α, IL-17 induces bone resorption in long bones in mice (Van bezooijen *et al*., 1999).

IL-17 has also been associated with the pathogenesis of periodontitis via induction of RANKL production by osteoclasts. Th17 cells and IL-17 have been detected in patients with chronic periodontitis and IL-17 levels were significantly higher in severe periodontal lesions compared with other T-helper secreted cytokines (Cardosa *et al.*, 2008). Takahashi *et al.* (2005)

reviewed its role in the immunopathology of periodontal disease and concluded that IL-17 is produced locally in periodontal lesion sites from activated gingival T cells. In the periodontal lesions, IL-17 induces the production of IL-6, RANK and RANKL, all of which are cytokines that increase bone resorption (Yamaguchi *et al*., 2012). Beklen *et al*. (2007) reviewed IL-17's role in bone destruction in periodontitis via a different cellular pathway – the upregulation of tissue-destruction matrix metalloproteinases (MMP). The authors concluded that IL-17 increases the presence of MMP indirectly by inducing IL-1β and TNF-α production in macrophages and IL-6 from gingival fibroblasts. Together, these cytokines regulate MMP which leads to the degradation of interstitial and membrane collagens.

In addition to bone resorption, IL-17 also pays a role in bone formation. Huang *et al*. (2006) stated that IL-17 is a growth factor for mesenchymal stem cells by stimulating the formation of colony forming unit-fibroblasts in marrow stromal cells. In another study by Kocic *et al.* (2012), IL-17 was shown to divert the differentiation of the myoblastic cell line C2C12 into an osteoblastic pathway. Both studies concluded that IL-17 is capable of steering the differentiation of mesenchymal stem cells into an osteogenic lineage.

Currently, IL-17's relationship with orthodontic tooth movement is not entirely clear but its role in rheumatoid arthritis and periodontitis as well as normal bone remodeling solidified its importance in bone metabolism. Studies have shown that excessive compressive forces during orthodontic tooth movement stimulate the expression of IL-17 which contributes to the osteoclastogenesis cascade (Nakano *et al*., 2015). Studies have also identified IL-17 as a pro-inflammatory cytokine for osteoclastic bone resorption during orthodontic tooth movement. IL-17 leads to the production of IL-1β, IL-6, and IL-8 which are all biomarkers for bone resorption (Gu *et al*., 2008). More recently, studies have linked IL-17 expression during orthodontic tooth

movement with a higher rate of root resorption via cytokine production of IL-6 to stimulate osteoclastogenesis and odontoclastogenesis. Higher levels of Th17 cells and IL-17 have been detected in bone slices exposed to higher orthodontic forces compared to lower orthodontic forces. (Hayashi *et al.*, 2011). Evidence suggests that IL-17 may be very important in tooth movement, but we do not fully understand its exact role. By evaluating levels of 1L -17 at various hours after force activation, we aim to learn more about its involvement and expression patterns during orthodontic tooth movement.

In this present study, the aim is to evaluate IL-17's presence in tension vs compression sites during orthodontic tooth movement. Since IL-17 plays a role in both osteoblast and osteoclast formation, it is hypothesized that its presence will be detected at both sites.

Gingival Crevicular Fluid

The gingival sulcus is a shallow crevice that is bound by tooth surface on one side and epithelial lining of the free gingival margin on the other. The fluid in the structure, called gingival crevicular fluid, (GCF) is an osmotically mediated inflammatory exudate that tends to increase in volume during tissue damage and inflammation from increased capillary permeability (Yamaguchi, 2009). Pashley (1975) concluded that increased GCF production is attributed to the increased rate of capillary transduction.

Waerhuag (1950) first studied the transforming anatomy of the sulcus during the course of periodontal disease. Since then, numerous studies have proven that GCF is a reliable medium to measure a multitude of biological markers and bacteria present in the oral cavity and PDL space (Griffiths *et al*, 1992). While GCF is primarily composed of serum with enzymatic and non-enzymatic components, the composition can be influenced by the surrounding gingival tissue and bacteria (Cimasoni, 1983). Inflammatory mediators increase capillary pressure to

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increase leakage of proteins into the interstitial fluid (Pashley, 1975). As such, GCF can be found to contain a variety of substances including immunoglobulins, microorganisms, toxins, cells, electrolytes and lysosomal enzymes (Yamaguchi, 2009). Since tissue remodeling in orthodontic tooth movement is initiated by the inflammation process, it has been observed that biomarkers of bone remodeling can be found within GCF as well (Lamster and Novak, 1992). Studies have shown that quantification and identification of cells and proteins in GCF can accurately reflect the immune and inflammatory pathways in patients with periodontitis and in patients undergoing orthodontic treatment (Lamster, 1992; McCulloch, 1994; Ren *et al.*, 2002; Ren *et al.*, 2007). During orthodontic tooth movement, increased levels of prostaglandin E, IL-1β, IL-6, tumor necrosis factor were reported in GCF (Grieve *et al*., 1994). GCF collection is a reliable non-invasive simple method to study a variety of biological markers without disturbing the integrity of marginal tissues.

GCF Levels of IL-1β and IL-17

While many studies have used GCF levels to study the inflammatory presence during active periodontal disease, fewer studies have examined similar markers during orthodontic tooth movement. Studies examining the levels of IL-1β in GCF during tension vs compression sites during orthodontic force application have limited time points at 1 and 24 hours after force activation. Grieve *et al.* (1994) found a peak increase in IL-1β after 24 hours of force application. Uematsu *et al.* (1996) has also found that GCF levels of IL-6, TNF-α, and epidermal growth factor were all significantly higher at 24 hours after force application during orthodontic tooth movement. However, these studies did not evaluate any time points between 1 and 24 hours post-orthodontic force activation. Presently, there are no studies characterizing the levels of IL-17 during orthodontic tooth movement comparing compression vs tension sites at

any time point. This present study aims to examine levels of IL-1β and IL-17 in GCF at 0, 1, 6 and 24 hours after orthodontic force application.

Summary

Orthodontic tooth movement is a complex biological phenomenon that involves a coordinated symphony of molecular signaling and interactions. This process has been documented by the four stages of tooth movement. Studies have shown that during the initial stages of bone remodeling, IL-1β is critical in initiating bone resorption via the activation of osteoclasts. This current study examines the presence of IL-1β and IL-17 in GCF during orthodontic tooth movement to determine if there is a correlation between peak levels of IL-1β and IL-17 and if they exhibit the same temporal expression pattern. The hypothesis is that the highest level of IL-1β detected in GCF occurs earlier than 24 hours after orthodontic force application. In addition, levels of IL-I7 in GCF will also peak prior to 24 hours and will follow a similar expression pattern as IL-1β.   This knowledge may help orthodontists with establishing activation intervals and may support the need for future studies to examine the mechanisms of accelerated orthodontic tooth movement. This knowledge will also help scientists understand more about the temporal behavior, possible similarities, and potential relationships of IL-1β and IL-17 in initiating orthodontic tooth movement, and examine if their expression and recruitment behavior is in concert or related.

Chapter III
Materials and Methods

Study Population

Gingival crevicular fluid samples were collected by Mathue Faulkner, DDS, MS from nine orthodontic patients [5 males, 4 females, age range from 11 to 31 years (mean 17.445 years)], treated at the University of Nevada, Las Vegas School of Dental Medicine Orthodontic Clinic. All patients were asked to participate in the study during their initial screening appointment and consent was obtained prior to sampling and beginning orthodontic treatment. Research protocol and consent forms were approved by the Office of Research Integrity – Human Subjects (02/22/11 by Faulkner). All patients participating in this study consented that their GCF samples could be used in future studies as well. IRB approval was obtained for this additional study using these previously collected samples (IRB exempt approval 11/8/2017 by Wen).

All patients were determined to be appropriate candidates to receive orthodontic treatment with fixed appliances. The inclusion criteria for this study consisted of: no self-reported use of anti-inflammatory medications, good periodontal health (generalized probing depths ≤ 3mm and no radiographic evidence of periodontal bone loss), and the presence of one or more teeth that did not require immediate orthodontic movement to serve as control teeth. Patient exclusion from this study included: presence of periodontal disease and/or moderate to severe gingivitis, only minimal required tooth movement, diabetes, self-reported use of anti-inflammatory medication within the last 30 days, history of bleeding problems, use of tobacco, and/or poor oral hygiene.

Data Collection

Pre-treatment records of intraoral and extra-oral photographs, panoramic and cephalometric radiographs, and study models were obtained for each patient. Before bonding, patient's oral and periodontal health was assessed. Probing depths, bleeding on probing (BOP), and a plaque index (PI) were performed and recorded at both initial and retie visits. The experimental tooth was chosen by evaluating the amount of predicted movement from initial models and ease sample collection. The experimental teeth consisted of five maxillary lateral incisors, three mandibular lateral incisors, and one maxillary central incisor (Table 1). Tension and compression sites were predicted from evaluating preliminary study models and calculating the direction of movement the tooth would undergo. The selected control tooth per patient was teeth that did not require great movement or immediate orthodontic bonding. Of the nine selected control teeth, one was a maxillary lateral incisor, one was a maxillary canine, one was a mandibular canine, two were mandibular incisors, two were mandibular premolars, and two were maxillary premolars (Table 1). The control teeth were not bonded, thus no force was applied until the completion of this study.

Table 1. List of Control and Experimental Teeth

Experimental Teeth	Count
Maxillary Lateral Incisor	5
Mandibular Lateral Incisor	3
Maxillary Central Incisor	1

Control Teeth	Count
Maxillary Lateral Incisor	1
Maxillary Canine	1
Mandibular Canine	1
Mandibular Central Incisor	2
Mandibular Premolar	2
Maxillary Premolar	2

GCF samples were collected before bracket placement at three separate sites for each patient. These sample sites were the mesiobuccal (MB) and distolingual (DL) of the experimental teeth as well as the mesiobuccal of the control teeth. Isolation was achieved with cotton rolls and teeth were carefully dried with air. Periopaper (Oraflow, Smithtown, New York) was gently inserted into the sulci to collect the GCF from each site for approximately 90 seconds. Samples were collected three individual times per site. The Periopaper was then placed in a Periotron 6000 (Oraflow, Smithtown, New York) to quantify total GCF volume collected from each site. The three Periopapers from the same site per patient were placed in a single vial diluted with 200 μL of 1X phosphate buffered saline (PBS). The vials were stored in a standard freezer until all samples were collected and processed. All vials were moved to a -20°C freezer on the same day and kept frozen until assay.

Following the initial sample collection (0 hrs), patients had brackets placed except for the control teeth. A 0.012 or 0.014 Nickel Titanium arch wire was used to align teeth and apply gentle orthodontic force. One hour after the wire was placed, GCF samples were collected in the same manner as the initial samples. At the 6 and 24 hours time point, GCF samples were also collected with the same protocol as the initial samples.

After 5-7 weeks from initial bonding, patients returned for their first retie appointment to re-activate orthodontic forces. Patients' periodontal health was assessed again by measuring probing depths, BOP, and PI. Exclusion criteria were reevaluated for each patient and no patients were eliminated from the study based on presence of periodontal disease and/or moderate to severe gingivitis and/or poor oral hygiene. GCF was collected after the periodontal exam prior to untying the wire or applying new orthodontic force. This represented the 0 hr time point at the retie appointment. Seven of the nine patients could progress to a thicker wire and had a 0.016 or

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0.018 Nickel Titanium arch wire placed. The initial arch wire was removed and retied for the remaining two patients since a larger wire could not be effectively engaged in the mal-aligned experimental teeth. Samples were again collected 1, 6, and 24 hours after orthodontic forces application using the same data collection protocol. All patient samples from the initial and retie visits were collected and processed by Mathue Faulkner (2011).

GCF Volume Measurement

To calibrate the Periotron 6000, 14 sequentially increasing known fluid volumes (0.1-1.4 µL) of fetal bovine serum (FBS) was added to Periopaper strips and placed in the Periotron for analysis. Each volume was tested and recorded three times to minimize error. The mean value per volume was calculated and a calibration curve was plotted using MasterPlex 2010 (Hitachi Solutions) analysis software to generate a conversion coefficient. A standard calibration curve using a fourth polynomial regression equation was formulated from the mean values, and was used to convert the samples from the Periopapers into volumes. The three volumes obtained per sample site were added together to calculate total GCF volume per sample.

GCF Protein Concentration

The protein concentrations in collected GCF samples were determined by a commercially available protein assay reagent based upon the Bradford method of protein concentration determination (Bradford, 1976). Each sample (20 µl) was combined with 275 µl of Protein Dye Reagent (Bio-Rad, Hercules, CA) after the microtiter plate assay as directed by the manufacturer. After a 10 minute room temperature incubation with shaking, the absorbance at 595 nm was determined using a SpectaMax Plus (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer. Bovine serum albumin was used as the protein standards for generation of

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the standard curve and unknown concentrations were calculated. All standards and samples were assayed in duplicate.

IL-1β and IL-17 Levels in GCF

IL-1β and IL-17 levels in GCF were analyzed with a MILLIPLEX MAP assays kit (Millipore, Billercia, MA) according to manufacturer's instructions. To prepare the samples for the multiplex assay, patient samples were thawed on ice and vortexed for 30 seconds, and sonicated for 30 seconds. Wash buffer (200 µL) was added to the 96-well plate and placed on plate shaker to be agitated for 10 minutes. Wash buffer was decanted and 25 µL of provided standard or control samples were added into appropriate wells. Assay buffer (25 µL) was added to background and sample wells. Millipore matrix solution (25 µL) was added to background, standards and control wells. Patient samples (25 µL) were added to corresponding sample wells. Beads (25 µL) were added into each well. The plates were then incubated overnight at 4 ºC on a plate shaker at 500rpm.

Table 2. Components Added to Each Assay Well

	Millipore Background	Millipore Standards	Millipore Quality Controls	Samples (Experimental and Control Sites)
Millipore Standards (IL-1β and IL-17)		25 µL		
Millipore Quality Control Samples			25 µL	
Assay Buffer	25 µL			25 µL
GCF Samples (experimental and control sites)				25 µL
Matrix Solution	25 µL	25 µL	25 µL	
Beads	25 µL	25 µL	25 µL	25 µL
Total µL in wells	**75 µL**	**75 µL**	**75 µL**	**75 µL**

After overnight incubation and agitation, well contents were removed by washing the plate 2 times with gentle vacuuming (BioTek, Winnoski, VT). Detection Antibodies (25 µL) were added per well and incubated for 1 hour on a plate shaker at room temperature. Strepavidin-Phycoerythrin (25 µL) was then added per well and incubated for 30 minutes on a plate shaker at room temperature. After 30 minutes of agitation, well contents were gently removed with a Bio-Tek ELx405 plate washer (BioTek, Winnoski, VT) twice. Sheath Fluid (150 µL) was added to all wells and the 96 well plate was placed on plate shaker to resuspend beads for 5 minutes.

The sample plates were analyzed on a Luminex 200 (Luminex Corp., Austin, TX). Raw data was exported from the Luminex 200 and standard curves and unknowns were analyzed using xPONENT software (Luminex Corp., Austin, TX, Version 3.1) with results expressed as fg/μg of total protein in GCF.

Statistical Analysis

The mean values for GCF volume, total protein, IL-1β and IL-17 for both experimental sites and the control site were compared using Student's t test using Microsoft Excel (2010). Significance was set at $p < 0.1$. Student's t-tests were performed comparing data within each time point, and across all time points.

Chapter IV

Results

Patient and Sample Characteristics

Five male and four female UNLV orthodontic patients, ages 11 to 31, were recruited to participate in this study (Table 3). At each time point, samples were collected from the mesiobuccal and distolingual of the experimental teeth and the mesiobuccal site of the control teeth during the initial and retie appointments. Periopaper (Oraflow, Smithtown, New York) was used to collect GCF samples and were then placed in a Periotron 6000 (Oraflow, Smithtown, New York) to convert fluids into volume measurements. This procedure was repeated three times per sampling site, and the three Periopaper samples for each site were placed in a single vial diluted with phosphate buffered saline (PBS). At the recall appointment, two sets of samples were not collected due to conflict with patients' schedules (Table 4).

Table 3. Patient Characteristics

Sex	n
Male	5
Female	4

Age	n
Mean	17.4
Max	31.3
Min	11.2

Table 4. Summary of GCF Samples Collected

	Post Activation (Hours)	Test Site MB (n)	Test Site DL (n)	Control Site MB (n)
Initial	0	9	9	9
	1	9	9	9
	6	9	9	9
	24	9	9	9
Recall (5-7 weeks)	0	9	9	9
	1	8	8	8
	6	9	9	9
	24	8	8	8
Total		70	70	70
				=210

Gingival Crevicular Fluid Volume

A standard curve was generated to convert Periotron readings for each Periopaper into GCF volume (μL). The standard curve was generated by analyzing readings from Periopaper absorbed with sequentially known volumes of fetal bovine serum (Figure 1). Recorded Periotron readings for each Periopaper were then converted into GCF volume for analysis and total GCF for each sample was calculated by adding together the individual readings for each of the 3 Periopapers that constitute a single sample.
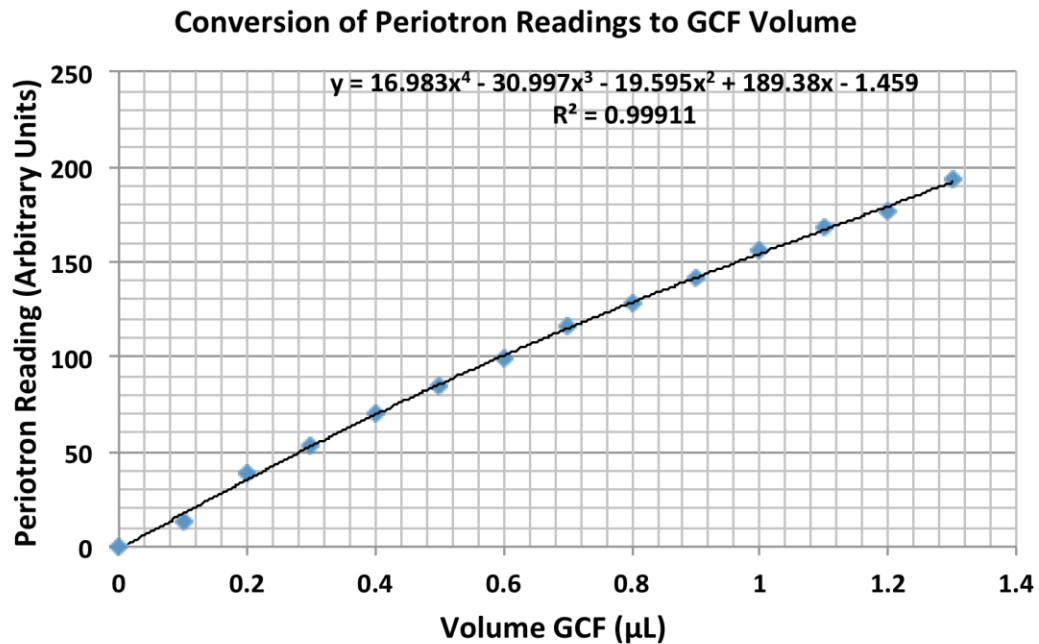
Figure 1. Conversion of Periotron Reading to GCF Volume (µL)
Figure from Faulkner, 2011

The GCF volumes were measured to determine if there were differences in volumes from each time point during orthodontic tooth movement. The measurements of GCF volumes for control and experimental sites (MB and DL) at each time point including both the initial (first) and recall (second) appointments are described in Figure 2. The GCF volume for control teeth ranged from 0.1 µL to 1.9 µL. The GCF volumes for experimental teeth had a greater range of 0.1 µL to 4.3 µL. Mean GCF volumes were calculated for each time point. The difference in amount collected between the mean GCF volumes of the experimental samples from the initial visit (1.13 ± 0.50 µL) and the mean GCF volumes of the experimental samples from the recall visit (1.05 ± 0.50 µL) was not statistically significant ($p=0.25$).   Both the control sample mean GCF volumes measured from the initial appointment (0.80 ± 0.22 µL) and recall appointment (0.85 ± 0.32 µL) were not statistically significant ($p=0.26$). However, when comparing the mean GCF volumes of the experimental samples to the mean GCF volumes of the control samples,

there was a statistically significant difference in volume size for both the initial appointment ($p=$ 0.009) and the recall appointment ($p=$ 0.05) (Figure 2).
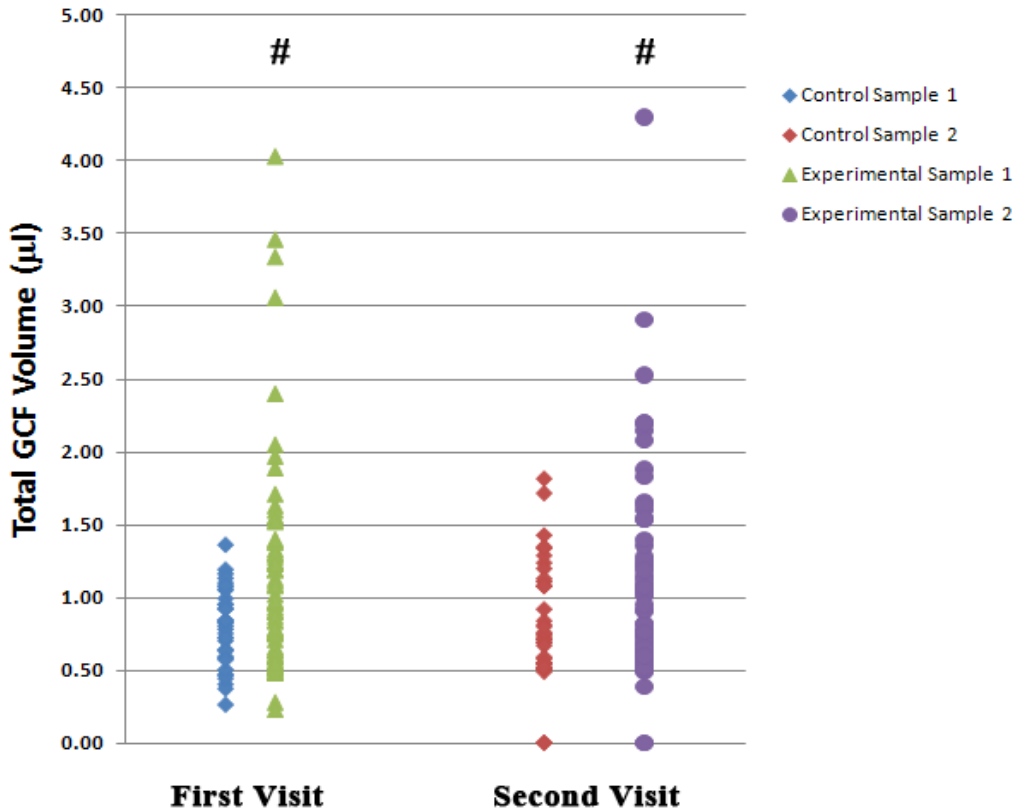
Figure 2. Total GCF Volume Collected During First and Second Visit
The difference in GCF volumes between the first and second visit for both control and experimental samples were not statistically significant. There is a significant increase in the amount of GCF collected from experimental teeth versus control teeth at both the initial and recall visits ($p< 0.1$). Figure from Faulkner, 2011.

During initial and recall appointments, there is a statistically significant increase in GCF volume at 6 hours compared to all time points from the experimental samples collected ($p<0.1$). The difference in experimental and control mean GCF volumes were statistically significant at the 1 hour ($p=0.08$), 6 hour ($p=0.02$) and 24 hour ($p=0.03$) time points. Experimental mean GCF

volumes were all higher than control mean GCF volumes at all time points after orthodontic

force was applied.   When evaluating the control mean GCF volumes across all time points, the

differences were not statistically significant (Figure 3).
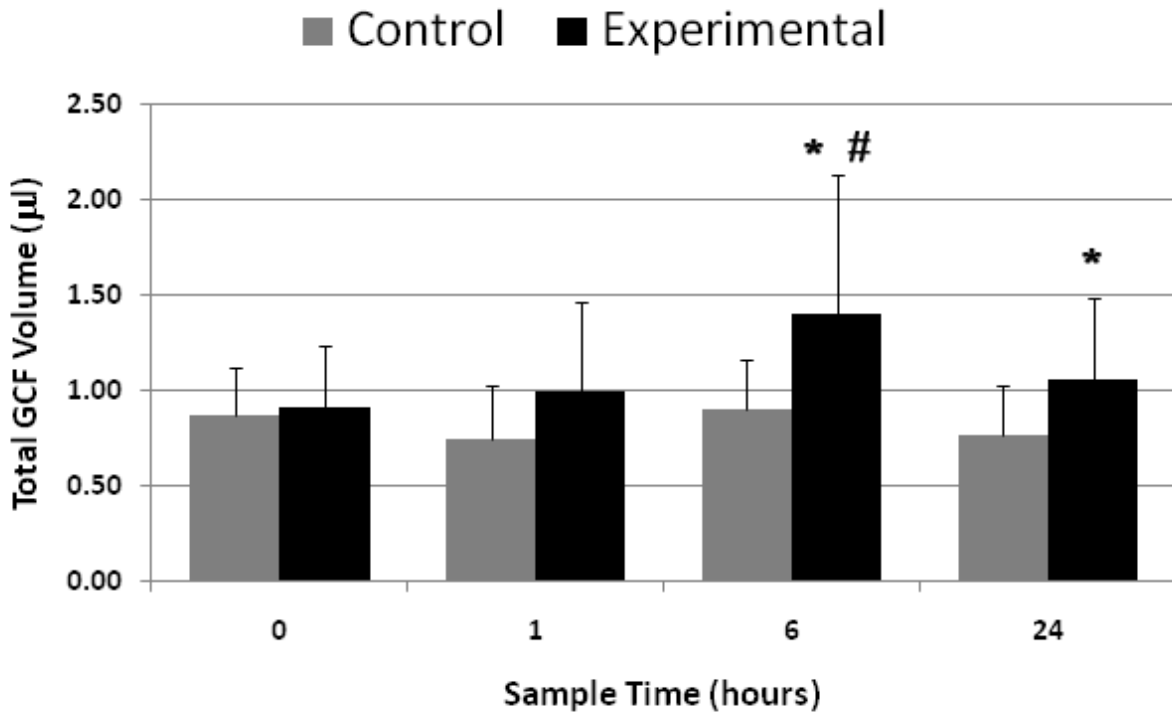
Figure 3. GCF Volumes Reported in Hours since Activation
The experimental mean GCF volumes had a statistically significant increase at the 6-hour time point compared to all other experimental time points ($p < 0.1$). There was a statistical significant difference in mean GCF volumes between experimental and control samples at 1 hour (*$p=0.08$), 6 hours (* $p=0.02$) and 24 hours (* $p=0.03$). Figure from Faulkner, 2011.

Gingival Crevicular Fluid Protein Levels

A Bradford assay (1976) was used to determine the protein concentration in each sample

of GCF. Known quantities of bovine serum albumin were used as protein standards and the

protein concentrations were plotted against the observed absorbance at 595 nm (Figure 4).
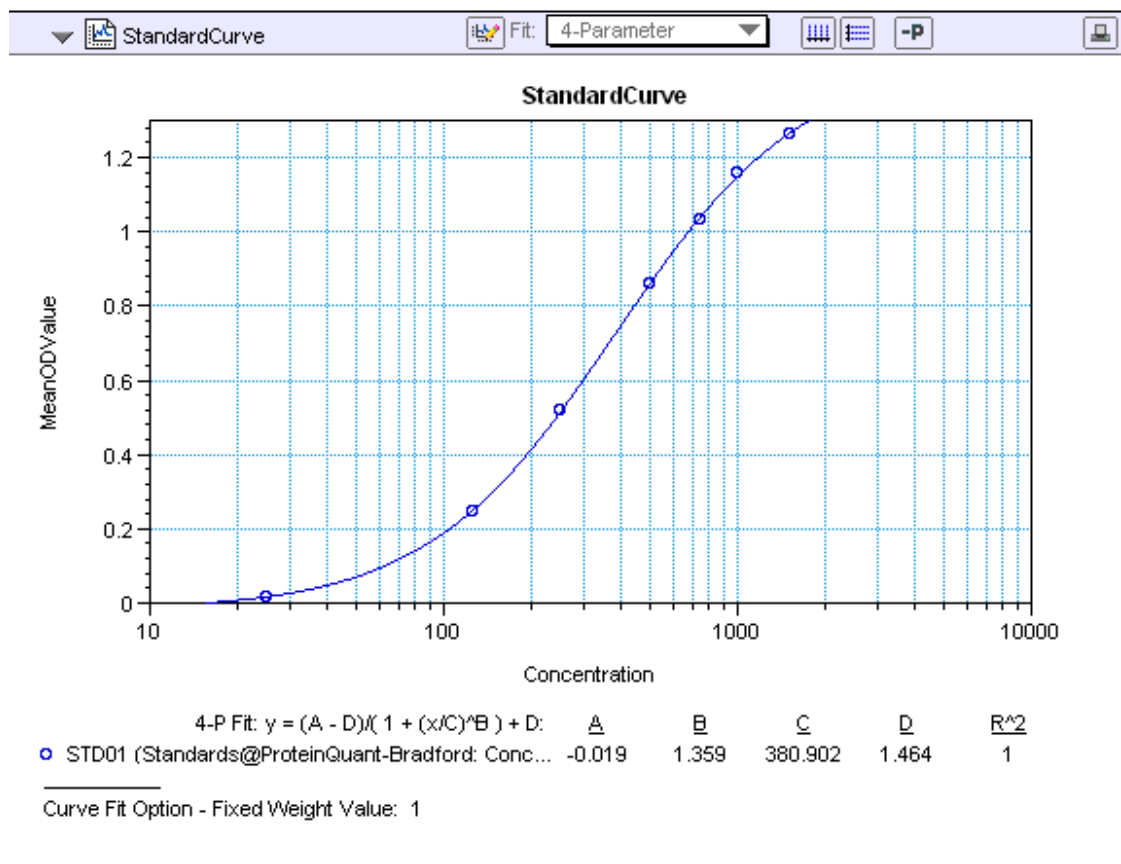
Figure 4: Standard Curve for Bradford Assay
Known quantities of BSA were measured at absorbance of 595 nm on a spectrophotometer (Molecular Devises SpectraMax) to create the standard curve. Bradford analysis was completed in duplicate with 20 μL of sample to determine the protein concentration (μg/mL) in eluted samples. Figure from Faulkner, 2011.

IL-1β Levels
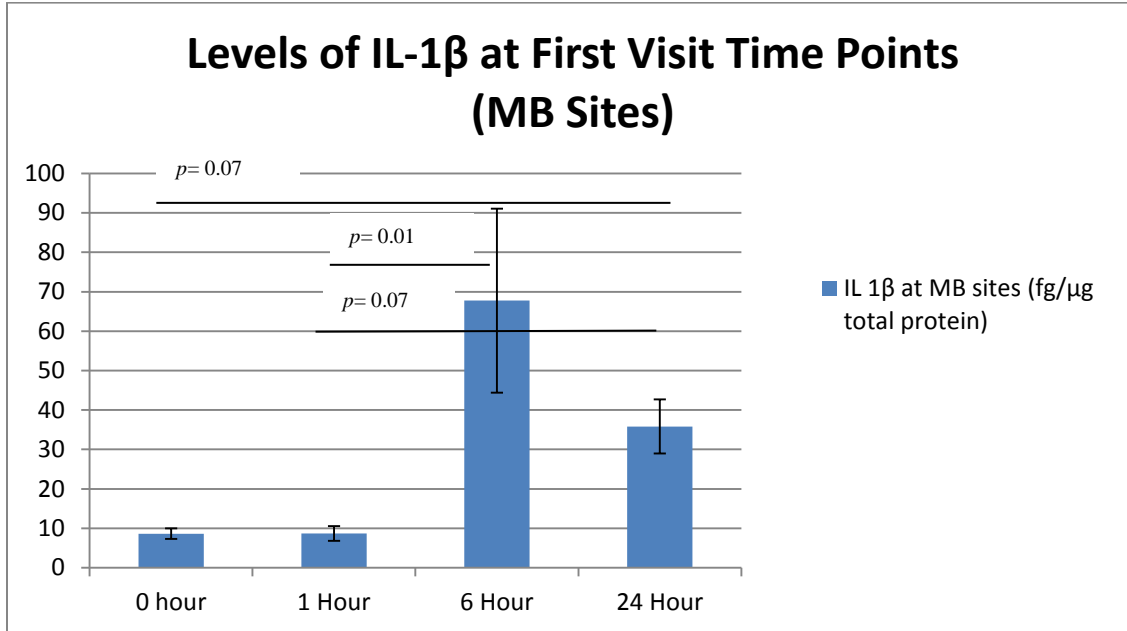
Figure 5: Levels of IL-1β at MB sites during Initial Visit Time Points
There was a statistically significant increase in IL-1β levels at 6 hours (p=0.01) and 24 hours (p= 0.07) on the MB of the experimental teeth relative to the 1 hour time point. Values are shown as fg of IL-1β per µg of total protein.

Elevated levels of IL-1β at the MB site were detected at 6 and 24 hours during the initial visit time points. The peak at 6 hours is greater than at 24 hours, suggesting that cytokine presence is greatest at 6 hours instead of 24 hours. IL-1β is elevated at 6 hours and this elevation is detectable up to 24 hrs. This is significant because this peak proliferation of IL-1β occurs hours earlier than previously thought. Differences in IL-1β levels were statistically significant between 0 and 24 hours (p= 0.07), 1 to 6 hours (p=0.01) and 1 to 24 hours (p= 0.07). However, the level of IL-1β between 6 and 24 hours is not statistically significant.
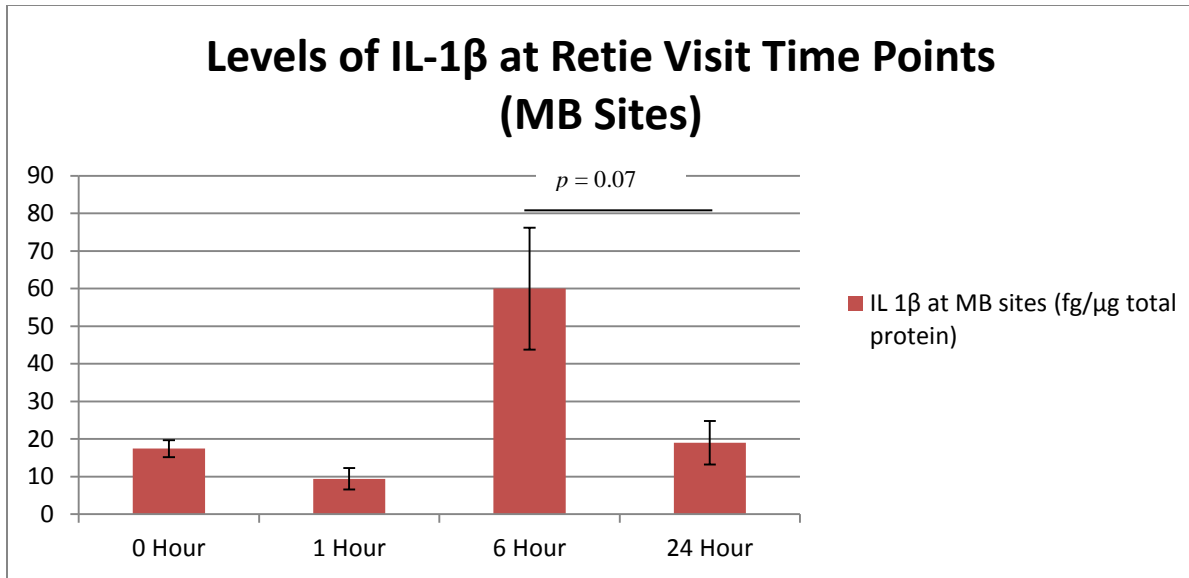
Figure 6: Levels of IL-1β in MB Sites during Retie Time Points
There was a statistical significant increase in IL-1β at the recall appointment between 6 hours and 24 hours ($p = 0.07$). Both initial and retie appointments show peak proliferation of IL-1β at 6 hours. Values are shown as fg of IL-1β per μg of total protein.

At the retie appointment, IL-1β exhibited a similar expression pattern as the initial appointment. From the graph above, levels of IL-1β were elevated at 6 hours and 24 hours compared to 0 hours and 1 hour. The peak at 6 hours was greater than 24 hours. At both the initial and retie time points, the expression of IL-1β peaked at 6 hours rather than 24 hours. The difference of IL-1β levels is statistically significant between 6 and 24 hours ($p = 0.07$).
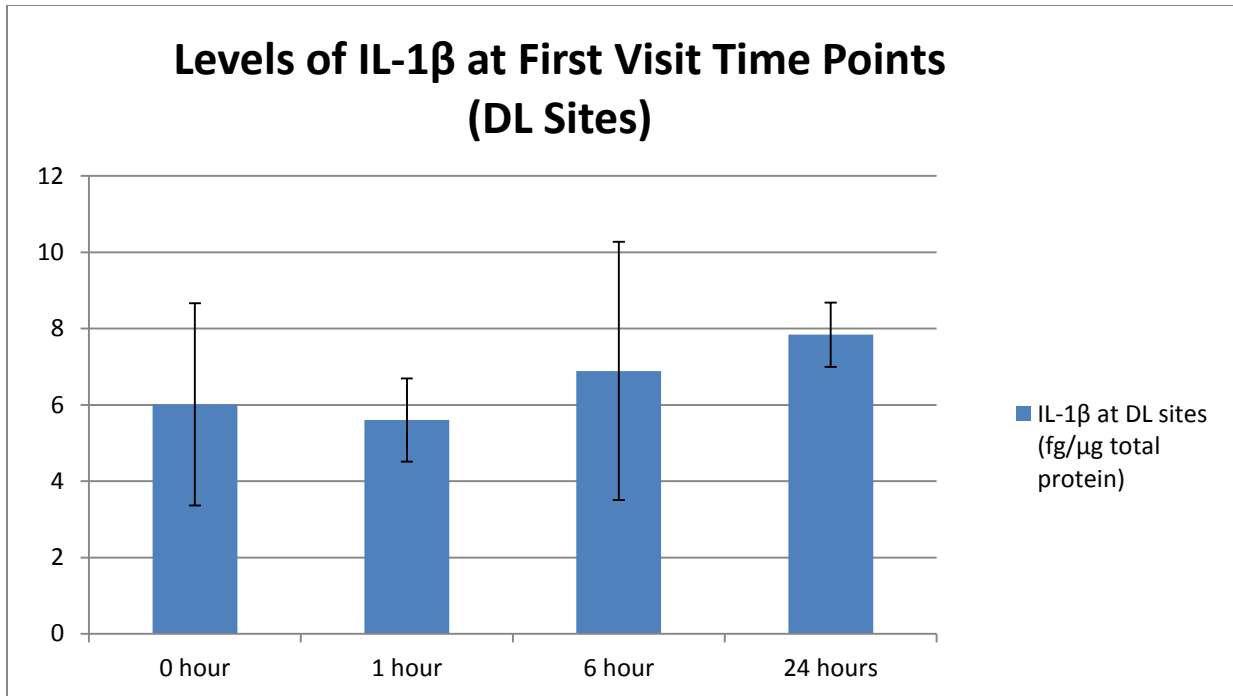
Figure 7: Levels of IL-1β at DL sites during Initial Visit Time Points
Although total IL-1β protein was highest at 24 hours at the DL site during initial visit, the differences when compared to the values at 0, 1, and 6 hours were not statistically significant. Values are shown as fg of IL-1β per μg of total protein.

When measuring IL-1β levels at the DL experimental sites during the initial visit time points, levels were elevated at 6 hours and 24 hours compared to 0 hours and 1 hour. The peak elevation of IL-1β was identified at the 24 hour time point instead of the 6 hour time point, as observed in MB sites. However, these differences in elevation at the DL sites are not statistically significant.

Previous studies have shown IL-1β to peak 24 hours after orthodontic force activation (Grieve *et al*., 1994). However, the study did not distinguish a tension and compression side, nor did they compare findings from the MB side to the DL side. At the DL site, this present study supports the findings that IL-1β peaks 24 hours after force activation.
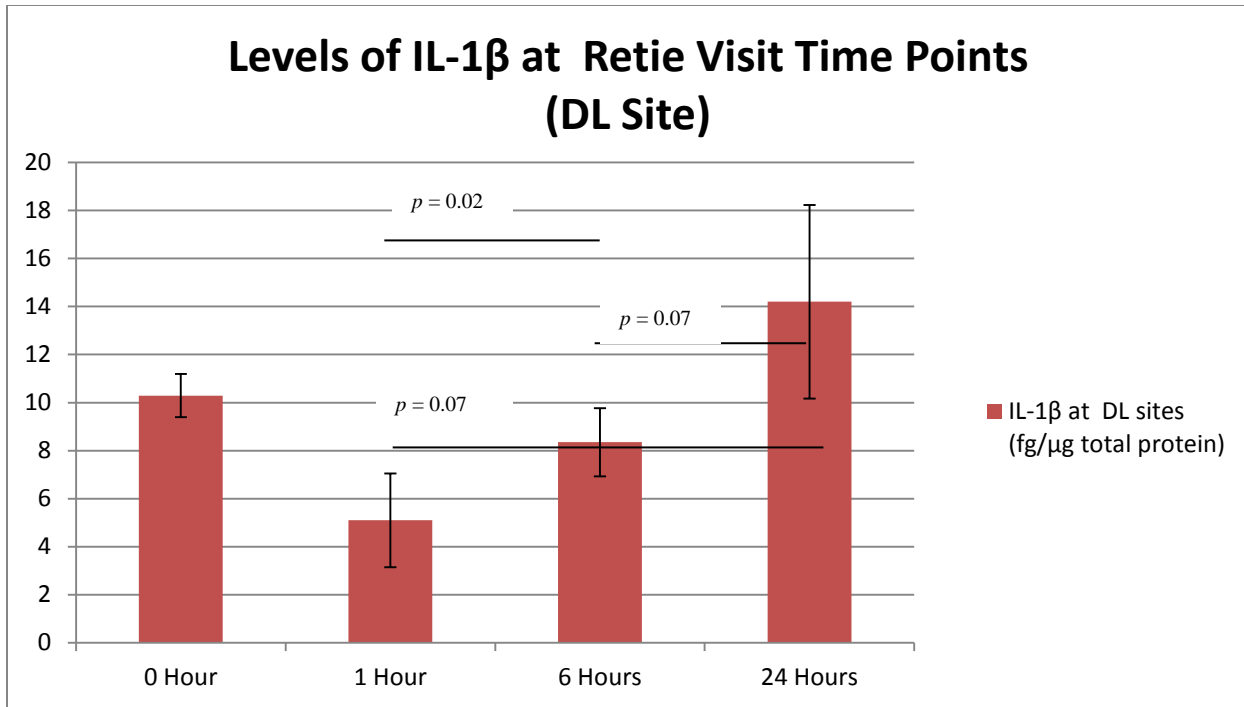
Figure 8: Levels of IL-1β at DL Site during Retie Appointments
At the retie appointment, IL-1β protein levels were highest at 24 hours compared to 0, 1, and 6 hours. The increase is statistically significant at 24 hours compared to 1 hour ($p= 0.07$) and compared to 6 hours ($p= 0.07$). Values are shown as fg of IL-1β per μg of total protein.

At the retie appointment, levels of IL-1β at the DL experimental sites were higher at 0 hours and 24 hours compared to 1 hour and 6 hours. The peak elevation of IL-1β was identified at 24 hours. The retie results slightly differed from the initial visit results as IL-1β was higher at 0 hours compared to 6 hours at the retie visit. However, both time points showed congruent data that IL-1β levels peaked at 24 hours post force activation. At the retie appointment, the differences in IL-1β levels measured at the DL sites were statistically significant between 1 and 6 hours ($p= 0.02$), 1 and 24 hours ($p= 0.07$), and 6 to 24 hours ($p= 0.07$).

IL-17 Levels
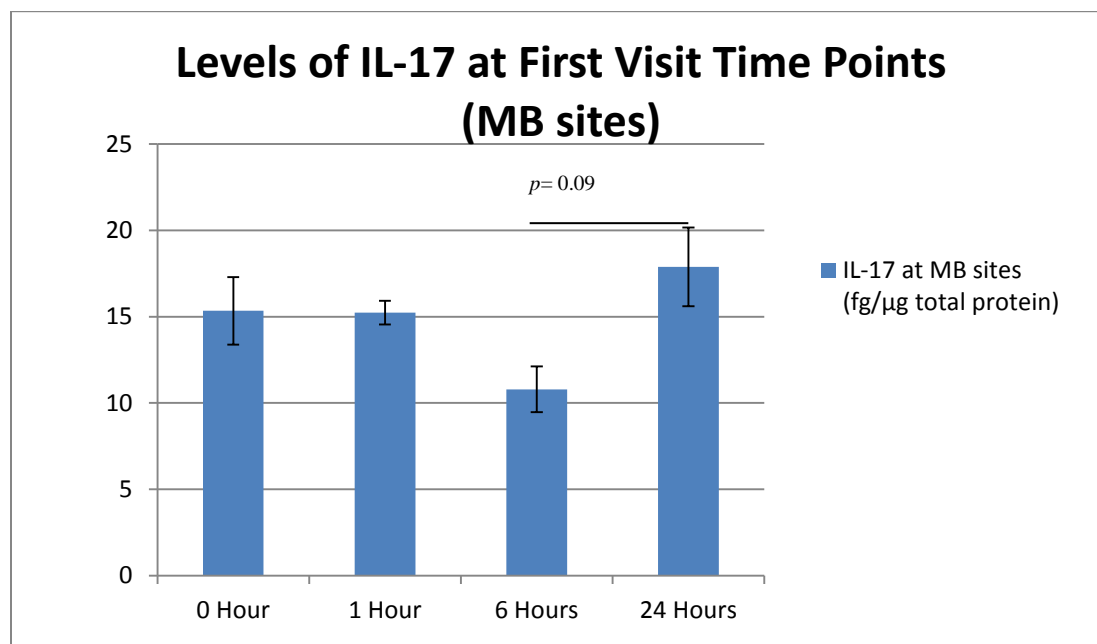
Figure 9: Levels of IL-17 at MB Sites during Initial Visit Time Points
IL-17 peaked at 24 hours after force activation and was statistically significant compared to levels at 6 hours ($p= 0.09$). Values are shown as fg of IL-17 per µg of total protein.

Levels of IL-17 peaked at 24 hours at the MB site during the first visit time points. Unlike IL-1β where the greatest levels were measured at 6 hours after force activation, levels of IL-17 at that time point were the lowest.   IL-17 levels was greatest at 24 hours but was not statistically significant when compared to 0, 1 hours.   The difference in measurements was statistically significant between 6 and 24 hours ($p= 0.09$).
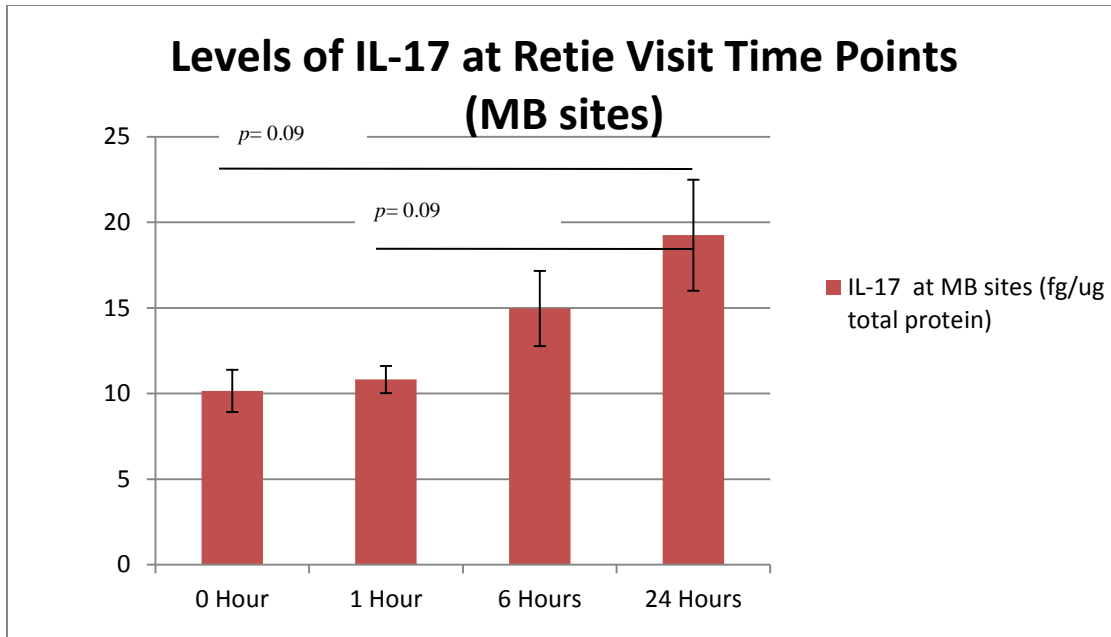
Figure 10: Levels of IL-17 at MB Site during Retie Appointments
At the retie appointment, levels of IL-17 at the MB site were highest at 24 hours. The peak was statistically significant when compared to 0 hours ($p= 0.09$) and compared to 1 hours ($p= 0.09$). Values are shown as fg of IL-17 per μg of total protein.

At the retie appointment, IL-17 levels were higher at 6 hours and 24 hours compared to 0 hour and 1 hour. The peak was measured at 24 hours. The peak following activation during the retie visit is consistent with the peak following activation at the initial appointment. This suggests that the peak expression of IL-17 occurs at 24 hours at the MB site during orthodontic tooth movement. The differences in IL-17 levels were statistically significant between 0 and 24 hours ($p= 0.09$) and from 1 and 24 hours ($p= 0.09$).
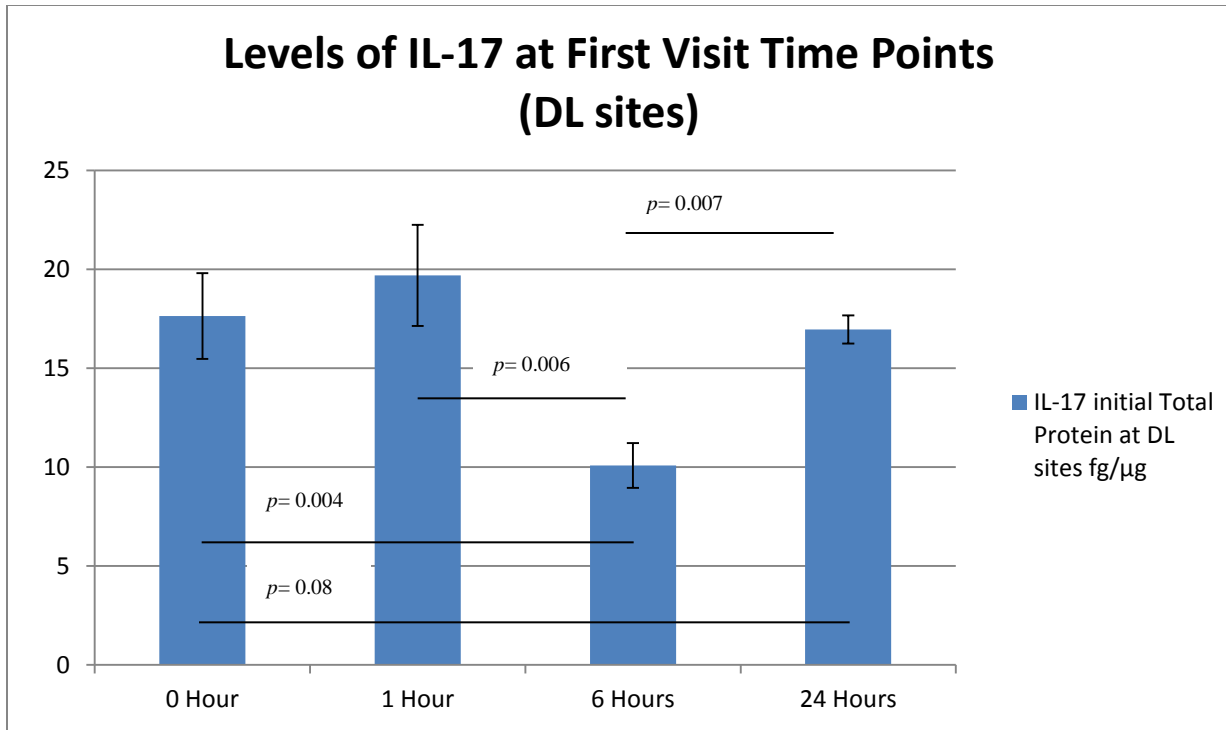
Figure 11: Levels of IL-17 at DL sites during Initial Visit Time Points
At the DL site, IL-17 peaked at 1 hour after force activation. The increase is statistically significant compared to 24 hours ($p= 0.01$). Values are shown as fg of IL-17 per µg of total protein.

Levels of IL-17 measured at the DL site during the initial visit time points did not exhibit a gradual increasing pattern of expression. Levels measured at 0 hour, 1 hour and 24 hours are higher than levels measured at the 6 hour time point. The peak was measured at the 1 hour time point. From the results gathered during the initial visit time points, there seems to be no temporal pattern of expression for IL-17 at the DL site. However, the differences in total IL-17 values were statistically significant between 0 and 6 hours ($p= 0.004$), 0 and 24 hours ($p= 0.08$), between 1 and 6 hours ($p=0.006$) and between 6 and 24 hours ($p= 0.007$).
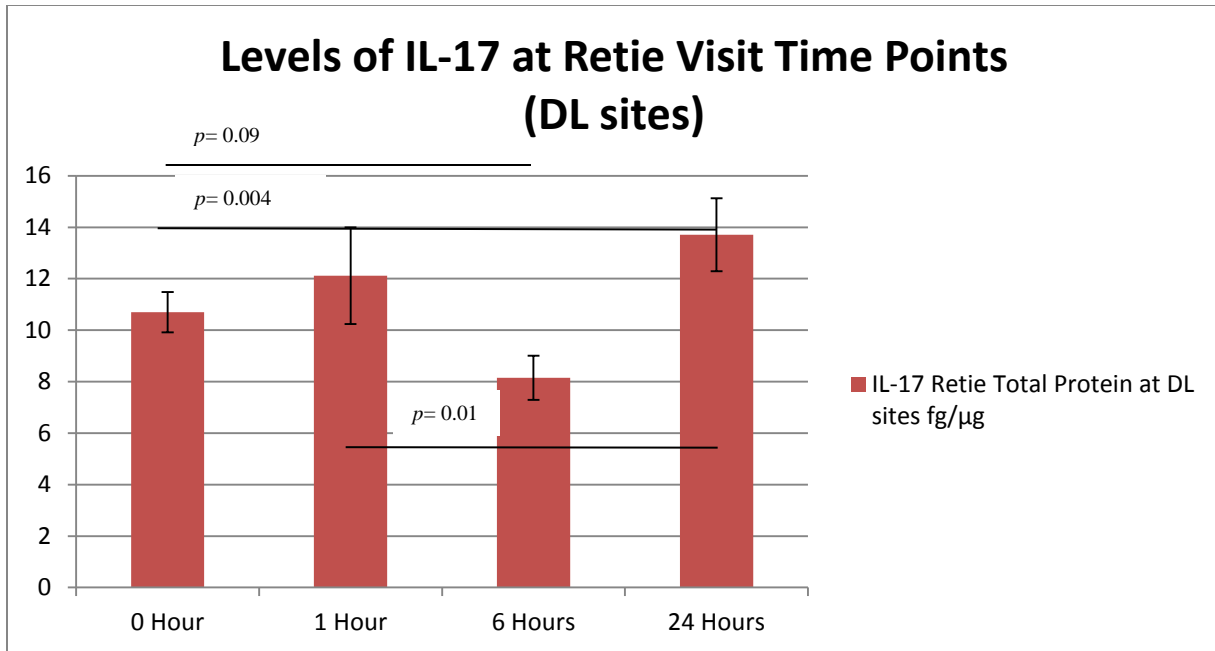
Figure 12: Levels of IL-17 at DL Site during Retie Appointments
IL-17 levels were highest at 1 hour after force activation. This corresponds to findings from the initial visit and was statistically significant compared to 24 hours ($p= 0.01$). Values are shown as fg of IL-17 per µg of total protein.

For the retie visit, there is also no gradual increasing pattern of IL-17 expression. Levels measured at 0 hour, 1 hour, and 24 hour time points were higher than at 6 hours. This is consistent with the findings from the initial visit that IL-17 levels were lowest at the 6 hour time point. Highest levels were measured at 24 hours. The retie results and initial visit results show no consistent temporal pattern of expression for IL-17. The differences in levels of IL-17 were statistically significant between 0 to 6 hours ($p= 0.09$), 0 to 24 hours ($p= 0.004$), and 1 to 24 hours ($p= 0.01$).

Chapter V

Discussion

Periodontal Condition

The periodontal conditions in our subjects were evaluated at initial and retie appointments. Although there was some slight marginal inflammation in localized areas due to position of brackets and hooks, no patients were excluded due to poor hygiene or severely inflamed tissue. This was attributed to subjects' good home care and oral hygiene. The interval between the initial and retie appointment was 5-7 weeks. The short duration of this study was selected to ensure optimal gingival health. Studies show that although it is achievable to maintain good oral hygiene throughout orthodontic treatment, duration in fixed appliances is an important factor in the health of gingival tissues (Skidmore et al., 2005).

All patients did not exhibit plaque accumulation on teeth or moderate gingival inflammation. Although our patients had good oral hygiene, it is possible that some levels of subgingival inflammation could not be detected clinically, which could account for higher levels of IL-1β and IL-17 in some samples.

Sample Age

The sample age in this study ranged widely from 11-31 years of age (mean 17.445). Although Ren *et al.* (2002) reported that cytokine levels (PGE$_2$, IL-6) in adolescents reflect changes in inflammation more accurately than cytokine levels in adults, information from both groups is important as demand for orthodontics from adult patients is increasing. Currently, there is little information on inflammatory mediators in GCF during orthodontic tooth movement in adults, thus future research can focus on comparing levels of IL-1β and IL-17 at different time points in different age groups and determine if cytokine levels vary with age.

Gingival Crevicular Fluid Volume

This study analyzed levels of IL-1β and IL-17 in gingival crevicular fluid after application of orthodontic force during different time points. GCF samples were collected from 9 patients undergoing treatment at the UNLV Orthodontic Clinic at their initial bonding appointment and at their first retie appointment. Baseline samples were collected prior to any application of force. Experimental samples were collected at 1, 6, and 24 hours after orthodontic force application for during the initial and retie appointments.

There was not a statistically significant difference in GCF volumes between the inital and retie visits for the control and experimental samples. However, when comparing control sample volumes to the experimental sample volumes, GCF volumes were higher in the experimental group.

Many studies have shown that GCF volume is a reliable and suitable source for biomarkers for tissue remodeling during orthodontic treatment (*Kapoor et al*., 2014). Many studies have also used GCF as the source for analyzing biomarkers and cytokine levels. In a systematic review completed by Kapoor *et al*. (2014), findings concluded that the altering levels of biomarkers in GCF corresponding to force application are indicative of the underlying biological remodeling processes in bone and periodontium during orthodontic tooth movement. RANKL, OPG, IL-1β, IL-6, TNF-α in GCF peaked at 24 hours after force activation (Kapoor *et al*. 2014) All of the studies analyzed in Kapoor's systematic review evaluated GCF samples collected 1 hour and 24 hours after force activation. Our study includes those time points in addition to evaluating samples collected at 6 hours after force activation.

There are also studies that show there is no statistically significant difference in GCF volumes of orthodontically treated teeth compared to control teeth, suggesting GCF cannot be used as an indicator of tooth movement. Drummond *et al*. (2011) conducted studies to evaluate if orthodontic tooth movement leads to significant changes in GCF volumes. They found that GCF volume is not a reliable method for evaluating tissue remodeling because changes in GCF volume were not statistically significant during orthodontic treatment. The small changes they saw between experimental and control teeth were attributed to subclinical tissue inflammation. These conflicting conclusions can be attributed to differences in patient population and oral hygiene, as well as study design and time points at which GCF samples were collected.

Since there was no statistically significant difference between the initial and retie appointments for GCF volumes within the same sample groups, further analysis examined GCF volumes by hours after force activation (0, 1, 6, and 24 hours). Experimental samples at 1, 6 and 24 hours showed higher GCF volume when compared to the control samples at every time point. In addition, GCF samples collected at the 6 hour time point from the experimental teeth showed a statistically significant increase in GCF volume compared to all other experimental time points. This is important as all other studies analyzing GCF volume during tooth movement have never measured volumes after 6 hours of orthodontic force application. The peak in GCF volume at this time point may suggest that inflammation associated with orthodontic tooth movement may also be at its peak. Since the 6 hour time point had the highest volume, further studies of GCF volumes at this time point may provide valuable information about the optimal time for assaying the level of inflammation induced by tissue remodeling.

Our study supports the conclusion that GCF volume does increase in teeth undergoing orthodontic treatment compared to control. The highest volume of GCF collected also

corresponds to the highest levels of IL-1β detected, both of which occurred at the 6 hour time point. This is significant because it shows that the elevation of IL-1β at 6 hours is not attributed to a relative decrease in total GCF volume. The elevation measured at the 6 hour time point is due to an increase in the expression of IL-1β.   This shows that GCF is a reliable source to evaluate cytokine levels and is reflective of the microenvironment of periodontal tissues.

IL-1β Levels

 IL-1β has been shown to induce osteoclastogenesis and play a critical role in bone resorption and apposition during orthodontic tooth movement. Previous studies of IL-1β in gingival crevicular fluid all analyzed its presence at 1 and 24 hours after force activation (Duduc *et al*., 2006). There are no studies analyzing IL-1β in tension vs compression sites collected from GCF at 6 hours post force activation. Therefore the general consensus has been that the peak expression of IL-1β occurs 24 hours after force activation.

Although other studies have suggested that peak IL-1β levels occurs 24 hours after force activation, this present study resulted in peak expression of IL-1β at 6 hours with a slight decrease at 24 hours. The decrease is statistically significant from 6 hours to 24 hours. This indicated that IL-1β peaks earlier than previously thought. Since no other study has evaluated IL-1β in GCF volume at this time point, IL-1β peaking at 6 hours may suggest that the inflammatory process is at its height during that time point. The inflammatory response is almost immediate after force activation, which possibly allows tooth movement to occur earlier. As tooth movement occurs earlier, clinicians could potentially shorten retie intervals and speed up treatment time.

The small GCF sample volumes (ranging from 0.1 to 4.3 μl) were diluted in PBS to a total volume of 200 μl so the concentration of our total protein in the working volume of PBS is

incredibly low. Some samples yielded no results when analyzed because the minimal detectable threshold of IL-1β was not present. Patient samples also exhibited highly variable readings of total protein, IL-1β and IL-17. Due to the high variability, our standard deviation was large, rendering many measured differences not significant. In many cases, there was a clear total value difference at each time point, but due to the high standard of error, some differences were not statistically significant. The wide range of results for some samples can be attributed to a plethora of reasons including patient variability, genetic differences, undetected subgingival inflammation, and varying force levels applied to teeth. Tooth movement depends on physical characteristics such as amount of force applied, as well as biological characteristics such as PDL size and genetic variability (Rygh and Brudvik, 1995). Thus each patient will most likely have varying rates of tissue response as evidenced by our samples.

IL 17

IL-17 has recently been identified as a cytokine involved in root resorption during orthodontic tooth movement (Aihara *et al*., 2013). Relatively little research has been done examining its role in bone turnover during orthodontic tooth movement. In a comprehensive review by Kumar *et al*. (2015), IL-17 was not listed as a known biomarker for orthodontic tooth movement. However, recent studies have shown elevated levels of IL-17 in patients with severe root resorption and concluded that IL-17 plays a role in root resorption by enhancing the expression of RANKL in PDL to initiate osteoclastogenesis (Nakano *et al*., 2015). Thus, a link has been made correlating IL-17 with bone remodeling during orthodontic tooth movement. To further investigate the role of IL-17, this present study examined the presence of IL-17 in GCF at several different time points.

In this present study, IL-17 peaked at 24 hours during both the initial and retie visits at the MB site. At the 6 hour time point, our initial and retie data are not analogous. At the initial appointment, levels at 6 hours were the lowest, while at the retie visit, levels at 6 hours were greater compared to 0 hour and 1 hour. Further research needs to be completed in order to establish a definitive timeline for the expression of IL-17.

Since IL-17 plays a role in both bone resorption and bone deposition, it was hypothesized that levels would be detected at both sites. At the MB site, IL-17 levels peaked at 24 hours during both the initial and retie appointments. However, our initial visit and retie data at the DL site are incongruous. During the initial visit, IL-17 levels peaked at 1 hour compared to 24 hours measured during the retie appointment. Our data for IL-17 at the DL site is inconclusive. This could be attributed to the assumption that IL-17 expression would theoretically be greatest during the post lag phase of tooth movement after removal of all necrotic bone by osteoblasts. IL-17 can divert the differentiation of mesenchymal stem cells into an osteoblastic pathway (Huang *et al*., 2006), which would mostly likely occur in the post lag phase characterized by increased osteoblastic activity. Since the study did not extend to 40 days post force activation, no conclusive data was gathered on the expression of IL-17 in DL sites. Further research needs to be conducted to evaluate IL-17's potential role in osteogenesis in orthodontic tooth movement.

From the raw data, levels of IL-17 were very similar across all patients and all time points. Though some differences between sites and time points were statistically significant, both sets of data showed similar total concentration levels. IL-17 has been detected at higher levels in patients with severe root resorption due to excessive orthodontic force and *in vitro* animal studies (Nakano *et al*., 2015). It could be possible that IL-17 is expressed in small quantities during healthy orthodontic tooth movement and levels only spike when excessive

force is applied. Since this study used slow gentle force application, high levels of IL-17 would not be expected to be detected. This can account for the generalized low levels of IL-17 present in GCF across all time points in our results. Low levels of IL-17 are needed for bone remodeling, but high levels might be associated with pathological bone remodeling that leads to root resorption. Higher levels of IL-17 are also detected in patients with periodontal disease. Thus low levels of IL-17 during orthodontic tooth movement could also be an indicator of periodontal health as well as root integrity.

Relationship between IL-1 β and IL-17

To determine if there is a relationship between the expression levels of IL-17 and IL-1β, this current study analyzed levels of the two cytokines in GCF during orthodontic movement at various time points. From our results, there is no temporal correlation between the expression of IL-17 and IL-1β. Hwang *et al* (2004) have shown IL-17 to induce the production of IL-6 and IL-8 in dental pulp cells. Although IL-6, IL-1β and IL-8 are pro-inflammatory cytokines that regulate bone remodeling, there appears to be no direct influence on the expression of IL-1β via IL-17. Currently, no literature has compared the expression levels of IL-17 to IL- 1β during orthodontic tooth movement. From our results, we conclude that IL-17 does not have a similar temporal expression pattern as IL- 1β and seems to have no temporal relationship with IL- 1β, but IL-17 can be used as a predictive factor for abnormal or aggressive inflammation.

MB and DL Sites

The present study investigated GCF from two opposite sites (mesiobuccal and distolingual) on the same experimental tooth. Samples from opposite sites were collected to analyze the cytokines present at the compression and tension sides of tooth movement. All of our experimental teeth were incisors, thus rotation and not translation was the projected movement

during the time frame of this study. From study models, it was predicted that bone resorption would more likely to occur on the MB side of the experimental teeth and was designated the compression site. The DL side was designated the tension site. However, because the movement of the tooth is mostly rotational and not translation, it was not possible to accurately designate either site as the purely a compression or tension side.   Despite this limitation, small differences were noted between the MB and DL sites. In measuring levels of IL-1β and IL-17, a statistically significant difference between the MB and DL experimental sites were identified for most time points. While it is difficult to accurately assign a pure tension and compression site, it is clear that orthodontic forces affect protein levels in resorption and apposition sites in varying magnitudes.

In the present study, IL-1β increased at 6 hours and 24 hours compared to baseline values and compared to control sites. Our observed changes in GCF composition support the theory that exogenous forces cause an inflammatory reaction at both the tension and compression sites. The differences in protein composition between the MB and DL sample sites may reflect the site specific recruitment of cytokines that occurs during orthodontic tooth movement.   However, there are no studies that analyze the site-specific sequential recruitment of cytokines, specifically between the MB and DL sites on the same tooth. GCF studies of cytokines during orthodontic movement only differentiate between an experimental site and a control site (Grieve *et al*., 1994). This limitation is exacerbated by the continuous nature of the PDL where GCF is free-flowing between all surfaces. This makes it difficult to attribute tissue remodeling biomarkers to one specific site in the PDL.

Initial vs Retie Visits

Nickel titanium wires are capable of withstanding large deflections and slowly return to their original shape on deactivation which translates gentle moderate forces to align teeth (Burstone *et al.*, 1985). Due to its high spring-back property and low load deflection rate, there might be some residual activation still present at 0 hours at the retie visit. This residual activation of the nickel titanium orthodontic wires could explain the elevated level of IL-1β at 0 hours at the retie visit compared to initial visit.

Future Research

Future studies can be performed with more time points and more patients. The sample size in this study was 9. A larger sample size could provide more conclusive and generalized data for all populations. Studies can focus on specific patient populations to gather data on specific groups such as male, female, age, ethnicity etc. More patients can also increase data points to garner more statistically significant results. In this study, we examined IL-1β and IL-17 expression at 0, 1, 6 and 24 hours after activation. It would be interesting to look at more time points between 1 hour and 24 hours activation since this present study is the first study to do so. GCF could be collected at 12 and 16 hours activation to complete a detailed expression timeline of IL-1β and IL-17.

If this study were to be conducted again, alternative methods could be used to increase detection sensitivity of IL-1β and IL-17 in our samples. Since our sample concentrations are very low and diluted further in the protocol, detection beads with greater sensitivity can help detect femtomolar cytokine levels and add to our data pool.

Other candidates associated with orthodontic tooth movement and tissue remodeling, such as TNF-alpha, IL-8, IL-16, can also be evaluated to analyze its temporal expression pattern.

Chapter VI

Conclusions

1. Gingival crevicular fluid volume increases after orthodontic forces are applied. This most likely is a reflection of the increased proteins and cytokine present in the periodontium due to inflammation from mechanical stimulus. In the present study, GCF fluid volume at the experimental sites peaked at 6 hours post force application and remained elevated at 24 hours post force application, suggesting that the inflammatory pathway is heightened during those time points.

2. IL-1β levels in GCF increased during orthodontic tooth movement at both the MB and DL sites and were highest at 6 hours post force application at the MB site. While all previous studies reported levels of IL-1β peaking at 24 hours, these studies did not measure levels between 1 and 24 hours. Levels of IL-1β peaking at 6 hours may imply that the inflammatory response is most reactive at 6 hours post force application.

3. IL-17 is detectable in the GCF during initial tooth movement. In contrast to the peak observed with IL-1β at 6hrs, IL-17 peaked at 24 hours at the MB site. It is possible that better detection methods or additional patients and sites may help to determine smaller variations in IL-17 at compression and tension sites. This may also help with defining a temporal relationship between IL-1β and IL-17.

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