Secretory Micro-RNA 29 in Gingival Crevicular Fluid During Canine Retraction

BY

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THESIS
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<table>
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<tr>
<td>3′-UTRs</td>
<td>3′-Untranslated Regions</td>
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<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone Sialoprotein</td>
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<tr>
<td>CTR</td>
<td>Calcitonin Receptor</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>GCF</td>
<td>Gingival Crevicular Fluid</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1 Beta</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>miRNA</td>
<td>Micro RNA</td>
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<tr>
<td>MMPs</td>
<td>Matrix Metallo-Proteinases</td>
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<td>OCN</td>
<td>Osteocalcin</td>
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<td>OCPs</td>
<td>Osteoclast Precursors</td>
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<td>OPG</td>
<td>Osteoprotegerin</td>
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<tr>
<td>OPN</td>
<td>Osteopontin</td>
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<tr>
<td>OTM</td>
<td>Orthodontic Tooth Movement</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>PDAC</td>
<td>Pancreatic Ductal Adenocarcinoma</td>
</tr>
<tr>
<td>PDCD4</td>
<td>Programmed Cell Death 4</td>
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<tr>
<td>PDL</td>
<td>Periodontal Ligament</td>
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<tr>
<td>PDLCs</td>
<td>Periodontal Ligament Cells</td>
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<td>PGE</td>
<td>Prostaglandin-E</td>
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<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>PLAP-1</td>
<td>Periodontal Ligament-Associated Protein-1</td>
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<td>RANKL</td>
<td>Nuclear Factor-κB Ligand</td>
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<td>RISC</td>
<td>RNA-Induced Silencing Complex</td>
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<td>TGF-β</td>
<td>Transforming Growth Factor-Beta</td>
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<td>TNFα</td>
<td>Tumor Necrosis Factor-Alpha</td>
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<tr>
<td>TRACP-5b</td>
<td>Tartrate-Resistant Acid Phosphatase 5b</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-Resistant Acid Phosphatase</td>
</tr>
<tr>
<td>UIC</td>
<td>University of Illinois at Chicago</td>
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SUMMARY

Gingival crevicular fluid (GCF) has been widely investigated as a potential source of biomarkers for an individual’s oral and general health information. In a healthy periodontal state, GCF is a serum transudate found in the crevicular sulcus and can be collected non-invasively. Changes in different substances found in GCF were reported in presence of orthodontic forces and various classes of molecules contained in GCF have been reported as potential biomarkers for orthodontic tooth movement (OTM). MicroRNAs (miRNAs) are non-coding RNAs that are involved in post-transcriptional gene regulation. MiRNAs downregulate their target genes’ expression primarily by base pairing with the messenger RNAs (mRNA), causing translational repression or mRNA degradation. Alterations in miRNA expression have been found to have a significant role in various diseases and tissue homeostasis. Recently, secretory miRNAs are being investigated as therapeutic as well as diagnostic tools.

In our laboratory, we discovered secretory miRNAs in GCF. In this study, we investigated potentials of certain miRNAs as biomarkers for detection of periodontal remodeling during OTM. The aim of the study was to confirm the presence of secretory miRNAs in GCF and investigate temporal expression profiles of specific secretory miRNAs during the course of orthodontic tooth movement.

A total of 70 subjects were recruited in the study from the patients receiving treatment at the University of Illinois at Chicago’s orthodontic clinic. The inclusion criteria included subjects requiring extraction of maxillary first premolars as part of their comprehensive treatment with edgewise fixed appliances. Due to gingival status changes and the patients’ inability to present at the specific time points for GCF collection, ultimately 11 GCF samples were collected from
11 healthy subjects (3 males and 8 females: 9 Hispanic, 1 African American and 1 Caucasian) between the ages 10 and 18 (mean 14.5 years old), who maintained excellent oral hygiene throughout the study. GCF was collected using absorbent Periopaper strips (OraFlow, Smithtown, NY).

GCF samples were collected at six time points during canine retraction as follows:

T0: prior to bonding the fixed orthodontic appliances

T1: on the day of canine retraction

T2: 60 minutes after activating the power chain

T3: 1 day after the canine retraction visit

T4: 7 days after the canine retraction visit

T5: between 5 weeks after initiation of canine retraction

The collected GCF was processed and analyzed. Human Let7d, g and i were used for normalization of secretory miRNA levels in GCF. Wilcoxon Sign Rank and Kruskal-Wallis and Mann-Whitney U statistical analyses were used in this study.

In all studied miRNAs, the change in expression of miRNA from T1 to T5 showed statistical significance consistently (p-value ranging from 0.005 to 0.047). Changes between T1 and T2 were only observed in miRNA-29b. Changes between T1 and T4 were observed in both miRNA-101 and miRNA-29b. Analyzing all the time points together, Kruskal-Wallis test showed no statistical significant difference between miRNA-29a, miRNA-29b and miRNA-29c,
(p>0.05, p-value ranging from 0.201 to 0.802) indicating the similarity in profile change of miRNA-29 family.

Comparison of responses between time points among the genes using Kruskal-Wallis detected statistically significant differences in $\Delta T1-T2$ (p-value= 0.012). Mann-Whitney U post-hoc test showed the observed differences were between miRNA-21 and miRNA-101, -29a and -29b ($p$-value = 0.004, $p$-value = 0.003 and $p$-value = 0.019 respectively). We concluded that secretory miRNAs exist in GCF and the expression levels of miRNA-29 family change during orthodontic tooth movement.
I. INTRODUCTION

A. Background

In 1892, German surgeon, Julius Wolff, suggested that bone remolds its structure in response to sustained mechanical forces (Frost, 2004). Since the introduction of Wolff’s law, the scientists’ understanding of the complex field of bone biology has evolved considerably, and throughout the course of this progression it has always been of significant interest to orthodontists. Orthodontic tooth movement (OTM) is accomplished by a relay of forces from orthodontic appliances to the periodontal housing of the tooth, which induces local molecular and cellular events, leading to periodontal and alveolar tissue remodeling. The effect of orthodontic stimuli on various cells and molecules of periodontal structures have been studied extensively. Identification of specific biomarkers for different stages of OTM would permit development of valuable clinical tools to assess phases and extent of periodontal and alveolar remodeling during treatment and the retention period, monitor for side effects of treatment such as root resorption and diminish patients’ pain experience. In addition, by considering the reaction of the individual patient to the treatment mechanics, the clinician can improve the efficacy of patients’ treatment by personalizing it to the individual and minimizing the side effects associated with orthodontic tooth movement.

Gingival crevicular fluid (GCF) has been widely investigated as a potential source of information regarding an individual’s oral and general health information. In the healthy periodontal state, GCF is a serum transudate found in crevicular sulcus and can be collected non-invasively. In periodontics, GCF biomarkers have been used as diagnostic tools to evaluate the periodontal health status of the patients. Changes in different
substances found in GCF can be detected in presence of orthodontic forces and various classes of molecules contained in the fluid have been studied as biomarkers for OTM. Our study focuses on a class of noncoding ribonucleic acids (RNA) called MicroRNAs (miRNAs) that are involved in post-transcriptional gene regulation. MiRNAs downregulate their target genes’ expression primarily by base pairing with messenger RNAs (mRNA), causing translational repression or mRNA degradation. Alterations in miRNA expression have been found to have a significant role in various diseases, and therefore miRNAs are being investigated as therapeutic as well as diagnostic tools. Key characteristics of miRNAs that make them good candidates as diagnostic factors are that they are secreted from the cell and exist as stable extracellular molecules, circulating in different body fluids, making them easy to collect.

Even though there have been numerous GCF constituent studies investigating changes in expression profiles of metabolites and substances involved in signaling and inflammatory cascades during OTM, no study reports the presence of miRNAs or investigated miRNA expression change in GCF in presence of orthodontic forces.

B. Specific Aims

The aim of the study was to investigate the expression profile changes of specific miRNA-29 family, miRNA-21 and miRNA-101 biomarkers during the course of orthodontic tooth movement. GCF samples of patients undergoing orthodontic treatment were collected at six time points through the course of treatment and analyzed. Associations between specific miRNAs and orthodontic tooth movement could potentially lead to new discoveries related to the gene regulation mechanisms involved in tooth movements. Determining the miRNA expression profiles associated with orthodontic tooth movement could also lead to development of clinically
valuable tools that allow for designing personalized orthodontic treatment for patients and improving the quality of orthodontic care.

C. **Objectives**

- Is secretory miRNA present in GCF?
- Does miRNA-29 family expression profile change during the course of tooth movement?
- Does miRNA-21 expression profile change during the course of tooth movement?
- Does miRNA-101 expression profile change during the course of tooth movement?
- Is the change related to the specific protein expression?

D. **Null Hypothesis**

Ho (1) - There is no difference between GCF miRNA levels in investigated time points before and after a directional force is put on the tooth.
II. REVIEW OF LITERATURE

A. Biological Response to Orthodontic Tooth Movement

Orthodontic tooth movement (OTM) is a biological process involving cellular and molecular events in connective tissues surrounding the dentition in response to mechanical forces applied by orthodontic appliances, leading to bone and periodontal ligament (PDL) remodeling and ultimately tooth movement. The tissue remodeling process is mediated via various cytokines, neurotransmitters and arachidonic acid metabolites (Krishnan and Davidovitch, 2015). Sandstedt was one of the first to visually demonstrate histologic tissue changes during OTM through light microscopy (Bister and Meikle, 2013). He employed a dog model to assess the areas of pressure and tension in PDL of incisors undergoing OTM and observed zones of bone resorption and apposition. Similarly, Reitan conducted histological examination of tissue surrounding the teeth with orthodontic forces applied to them, and found areas of hyalinization and necrotic tissue at the site of compression and zones of new trabeculation in the areas of tension (Krishnan and Davidovitch, 2015). Such observations lent support to the Pressure-Tension theory initially proposed by Shwarz (1932), which was traditionally used to explain biological bases of OTM (Masella et al, 2006; Storey, 1973).

Advances in cellular and molecular biology through evolution of investigative tools such as electron microscopy and progress in fields of histochemistry and immunohistochemistry, allowed bone biologists and orthodontic researchers to focus on the microscopic changes during OTM. Evidence of resorption lacunae and increased osteoclastic activity illustrated active participation of cells of hematopoietic lineage in tissue remodeling (Rygh, 1974). Macrophage activity was detected in removal of the necrotic tissue in the areas of compression during early stages of treatment (Krishnan and Davidovitch, 2015). The increased number of osteoclasts and
their precursors in areas of compression was observed in rats by Kawarizadeh (2004). Osteoblasts and osteocytes were found to be involved in both resorptive and appositional phases (Sandy et al., 1993).

The PDL regulates the remodeling of bone matrix in response to prolonged orthodontic forces by signaling the surrounding key cells in tissue remodeling such as PDL fibroblasts, osteoblasts, osteoclasts and osteocytes (Lekic and McCulloch, 1996). The tissue remodeling is largely dependent on the resorptive and appositional mechanisms in the periodontium extracellular matrix (ECM) (Kyrkanides et al., 2000). Extracellular matrix consists of a majority of collagenous matrix and a portion of non-collagenous matrix such as proteoglycans. The cellular component includes fibroblasts, endothelial cells and macrophages (Krishnan and Davidovitch, 2015). Altered collagen synthesis by PDL fibroblasts in response to orthodontic stimulation has been shown (Rygh, 1973; Bumann et al., 1997). Endothelial cells of extracellular matrix are involved in remodeling of ECM by their ability to contribute to remodeling of periodontal vasculature and their ability to produce matrix metalloproteinases (MMPs) and synthesize collagen (Kyrkanides et al., 2000). MMPs are protease enzymes produced by different types of cells including fibroblasts and endothelial cells, that are involved in degradation of collagenous portion of ECM (Sodek and Overall, 1992). Changes in other enzymes associated with bone remodeling, including acid phosphatase, lactate dehydrogenase and prostaglandin synthetase in experimental tooth movement in rat models have also been reported (Lilja et al., 1983).

The identification of receptor activator of nuclear factor-κB ligand (RANKL)-osteoprotegerin (OPG) pathway and its essential function in the regulation of bone metabolism in late 1990s helped advance the evolving understanding of biology of OTM. OPG was discovered
when transgenic mice overexpressing a gene, developed osteopetrosis due to lack of osteoclasts. The gene was appropriately named osteoprotegerin or bone protector (Simonet et al., 1997). While OPG inhibits osteoclastogenesis, RANKL, a protein secreted by osteoblasts, is involved in promoting the osteoclast formation by binding its receptor on osteoclast precursors (OCPs) and facilitating osteoclastogenesis (Yasuda et al., 1999).

RANKL’s promotion of osteoclastogenesis is mediated by prostaglandin E2 (PGE2). PDL cells under compressive force show increased PGE2 expression. Kanzaki et al. (2002) proposed that mechanical forces on PDL cells led to PGE2 synthesis which upregulated RANKL expression and induced osteoclastogenesis.

Zhang et al. (2004) demonstrated a change in RANKL/OPG expression ratio in human PDL cells under resorptive conditions. Vitamin D3, a hormone that promotes resorption, was introduced in human PDL cell cultures and changes RANKL/OPG mRNA expression was measured. Cells cultured in presence of vitamin D3 had reduced expression of OPG and increased expression of RANKL. The increase in RANKL/OPG ratio supports the notion that these molecules are important in regulation of bone metabolism.

Since osteoclasts are essential in OTM, changes in their activity can influence the rate of tooth movement. Numerous approaches have been reported to manipulate the efficiency, extent, and stability of the OTM. Topical and systemic administration of bisphosphonate, an osteoclastogenesis blockers, in rats showed a decrease in numbers of osteoclasts and the rate of tooth movement decreased in a dose dependent manner (Igarashi et al., 1994).

Compression of PDL results in blood flow modifications that lead to release of inflammatory mediators and signaling molecules. Davidovitch used immunohistochemistry
techniques to study changes in cell signaling molecules such as cytokines and growth factors during OTM in cats (Krishnan and Davidovitch, 2015). Increased production of proinflammatory factors such as interleukin-8, interleukin-6 and tumor necrosis factor-alpha (TNF-α) during OTM supports the fact that inflammatory pathways are involved in initiating tissue remodeling in OTM (Alhashimi et al., 2001; Ogasawara et al., 2004). The magnitude of expression of molecules involved in inflammatory process is correlated with the efficiency of bone remodeling. Yoshimatsu et al. (2006) showed a decreased rate of tooth movement and decrease in osteoclast activity in in TNF-α type 2 receptor-deficient mice. Studies have also shown that anti-inflammatory substances have a negative effect on the rate of tooth movement (Arias and Marquez-Orozco, 2006).

Baloul et al. (2011) examined rate of tooth movement in presence of corticotomy-induced osteoclastogenesis while monitoring the expression levels of key factors involved in regulation of osteoclastogenesis in rats. The animals that received selective alveolar decortication had an increased rate of tooth movement and increased osteoclastic activity as evident by an increased expression of RNA markers of osteoclastogenesis regulators: RANKL, OPG, calcitonin receptor (CTR), tartrate-resistant acid phosphatase 5b (TRACP-5b), cathepsin K and macrophage colony stimulating factor (M-CSF). The study also showed an increased expression of osteoblast activity markers such as osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN) in the response to the corticotomies and OTM.

Teixeira et al (2010) studied effects of osteoperforations on expression of 92 different cytokines and receptors during OTM in rats. Osteoperforations increased expression of 21 inflammatory cytokines and tartrate-resistant acid phosphatase (TRAP) staining corresponding to increased osteoclastic activity. The osteoperforation group demonstrated increased rate of tooth
movement with high levels of cytokines and osteoclast activity. This finding is consistent with the concept that inflammatory cytokines are involved in facilitating recruitment of osteoclasts and initiation of bone remodeling process (Alhashimi et al., 2001; Ren and Vissink, 2008; Krishnan and Davidovitch, 2006).

B. Biomarkers in Gingival Crevicular Fluid

Gingival crevicular fluid (GCF) is a serum transudate or inflammatory exudate in the gingival crevice or periodontal pocket (Last et al., 1988). GCF was identified when Brill and Krasse injected fluorescein dye intravenously into a dog and were able to collect the dye in the gingival crevice, an observation that gave hints as to the origin of the fluid. The fluid found in a healthy crevice is a transudate of interstitial fluid, whereas in presence of inflammation, GCF is an exudate that reflects serum content concentration (Brill and Krasse, 1958). GCF and its content have been evaluated for use as diagnostic biomarkers, substances that can be examined objectively to evaluate physiological, pathological processes, or responses to therapeutics (Griffiths, 2003).

GCF is considered an excellent tool for detection of diseases due to its convenient and noninvasive collection method and the clinician’s ability to perform multiple sampling from the same area using different techniques such as filter paper strips or micropipettes. In addition, unlike other body fluids, which are systemic in origin, GCF content can be used to evaluate the disease status of a localized site. As GCF makes its way from the vasculature, through the periodontal connective tissue, into the crevicular area, it acquires metabolites such as inflammatory mediators and antibodies present in its path, making its content a reflection of physiology of the area from which it has been collected (Last et al., 1988).
Ever since Brill first proposed using GCF to quantitatively evaluate the health status of periodontal tissue, there have been various studies to investigate the potential of GCF as a diagnostic tool for detection and diagnosis of periodontal disease. Studies examined GCF from diseased periodontal sites for presence of biomarkers of inflammation and periodontitis including host derived enzymes, cellular immune-response indicators and tissue breakdown products.

Changes in concentration of factors from all the categories were evident (AlRowis et al., 2014). Armitage et al. (2004) reports 65 constituents of GCF have been evaluated as potential markers for periodontal disease. Offenbacher et al. (1986) found that in adults with periodontitis, PGE2 levels in GCF can be used as indicator to differentiate between sites that are at risk of attachment loss and sites in remission state. Mogi et al. (2004) examined in vivo levels of RANKL and OPG in GCF of patients at different stages of periodontal diseases and found increased RANKL to OPG ratio in patients with periodontal disease as compared to healthy subject in the control group. The data suggested that the increased RANKL/OPG ratio in GCF could be potential biomarker for periodontitis.

In addition, some of the substances evaluated in GCF as biomarkers of periodontal inflammatory status contribute to OTM due to their roles in inflammation and tissue turnover. To date there have been many GCF studies investigating changes in expression of substances associated with bone remodeling, in search of biomarkers of orthodontic tooth movement. The ultimate goal of such studies is to find biomarkers that can be linked to relevant clinical phenomena to improve orthodontic treatment (Alhashimi et al, 2001; Kapoor et al., 2014).

Preliminary studies showed that several inflammatory and signaling substances responded to application of orthodontic forces. Grieve et al. (1994) used radioimmunoassays to
detect elevated expression of prostaglandin-E (PGE) and interleukin-1B (IL-1b) in early phases of orthodontic tooth movement in GCF of their subjects. Lowney et al. (1995) found a more than two-fold increase in TNF from the GCF after application of force. Uematsu et al. (1996) reported increases in concentration of IL-1β, IL-6, TNFα and epidermal growth factor in GCF of patients, 24 hours after application of force. Perinetti et al. (2002) investigated changes in alkaline phosphatase (ALP), an osteoblast activity marker, in GCF during OTM. The study reported detection of higher levels of ALP activity in the areas of tension as compared to the areas of compression. Nishijima et al. (2006) examined the changes in levels of RANKL and OPG in GCF collected from canines undergoing retraction at 0, 1, 24 and 168 hours after force application. They found a significant increase in RANKL and a decrease in OPG levels in GCF of experimental canines compared to the controls, 24 hours after initiation of retraction.

In addition to release of various metabolites, application of the force on the tooth causes an increase in vascular permeability in periodontium and since GCF is close to the site of the activities, it has great diagnostic potential for biomarkers of these processes (Rody et al., 2011). Alikhani et al. (2013) was able to demonstrate that the level of constituents detected in GCF reflect the extent of inflammatory stimuli. The investigation used a split-mouth design to study the effects of microperforations on relative expression of inflammatory biomarkers in OTM, and was able to measure significantly increased level of biomarkers in the side of the subjects’ mouth with osteomicroperforations.

Generally, to investigate relative expression of biomarkers, study designs have been such that there is a sampling of GCF prior to application of orthodontic stimuli, followed by sampling at multiple time points after the application of force. The predominant observation has been detection of peak levels of substance at a period of one or two days after the application of the
force and a gradual return to initial expression levels in about 7 days (Krishnan and Davidovitch, 2015).

C. **Secretory MicroRNA**

Micro-Ribonucleic Acid (miRNA) is a short non-coding ribonucleic acid (RNA) that is involved in post-transcriptional regulation of gene expression. The earliest report of miRNA was discovery of negative regulation of production of LIN-14 protein in C. elegans by a small antisense complementary RNA called lin-4 (Lee et al., 1993). This small RNA transcript did not code any proteins and it was found to contain a sequence complementary to the messenger RNA transcribed from the LIN-4 protein. In addition to being transcribed from their own genes, since miRNAs are non-coding RNAs, they can be transcribed from introns.

Precursors of miRNAs (Pri-miRNAs) are transcribed as a long primary transcript by RNA polymerase II. Pri-miRNA is processed by Drosha and DGCR8 in nucleus and the resulting pre-miRNAs is exported to the cytoplasm by exportin-5 protein (Murchison and Hannon, 2004). In the cytoplasm the hairpin structured pre-miRNA is cleaved by an endoribonuclease called Dicer resulting in a double stranded miRNA of about 22 nucleotides in length (Lund and Dahlberg, 2006). One strand of a mature miRNA is sufficient to regulate the messenger RNA through forming a ribonucleoprotein called a RNA-induced silencing complex (RISC) (Rana, 2007). The activated miRNA complex binds the complementary sequence on the target mRNA and the extent of base-pairing determines the fate of target mRNA; Perfect complementation leads to degradation of target mRNA whereas partial complementation leads to inhibition of translation of mRNA (Figure 1). The former is generally true in plants where miRNA binds to near perfection to the complementary sequence on either the coding or 3′-
untranslated regions (3′-UTRs) of the mRNA. In contrast, in animals, the miRNA commonly binds to many partially complementary sequences of the 3′-UTRs of the target mRNA (Bartel et al., 2009).

Figure 1. Biogenesis of microRNA

Since partial complementation of miRNA with the target mRNA is sufficient in its regulation, one miRNA is able to influence the expression of multiple genes. There are already more than 2500 miRNAs that have been reported in human by miRbase v21, it is estimated that one third of genes in human are affected by the miRNAs. Due to miRNAs' expansive role in gene expression regulation, their malfunction has been associated with various diseases including cardiomyopathies, neuropathies, hematological, metabolic and developmental diseases (Bartel, 2004). Oral biology researchers have also been exploring the regulatory role of miRNA in areas such as dental development, oral cancer and pulp inflammation and periodontal disease (Kim et al., 2015; Chan et al., 2013; Cao et al., 2010).
Several characteristics of miRNAs make them ideal candidates as diagnostic biomarkers. MiRNAs are secreted in extracellular space and can be detected in circulatory form. They have been isolated from more than 12 different types of body fluids, a feature that makes them easy to access and collect (Weber et al., 2010). In addition to being easily retrievable, circulating miRNA show exceptional stability, another prerequisite for an ideal biomarker. Studies monitored changes in human plasma miRNA, exposed to boiling temperature, multiple freeze-thaw cycles, high range of pH, and long time storage in room temperature, showed miRNA to be minimally affected by the conditions (Mitchell et al., 2008; Chen et al., 2008). The remarkable stability of circulating miRNAs can be explained by their association with microparticles such as exosomes, RNA binding proteins such as Argonaute2, and lipoprotein complexes such as high-density lipoprotein which protect the free floating miRNAs from degradation (Bartel, 2004).

The mechanical loading from orthodontic appliances relays signal transduction through the PDL and elicits a response from the bone (Krishnan and Davidovitch, 2006; Qian et al., 2001). Tooth movement occurs when alveolar bone and periodontal tissue remodel by an increase in expression of anabolic ECM genes in the tensile areas and a decrease in the anabolic activity in the compression side (Meikle, 2006; Xu et al., 2014). miRNAs are known to have regulatory roles in inflammatory cascade and ECM maintenance and remodeling, two principal processes involved in tissue remodeling (Rutnam et al., 2013). MiRNAs are also involved in transduction of mechanical signal pathways and studies have already shown that miRNA expression responds to changes in the mechanical environment of cells and therefore are likely to be involved in regulation of molecules associated with orthodontic movement (Chen et al., 2015; Mouw et al., 2014).
Chen et al. (2015) investigated force-induced alterations in expression levels of miRNA-29 family in human PDL cells and found that miRNA-29 family expression responded to the orientation of induced forces. MiRNA-29 family has been reported to be involved in regulation of several ECM proteins and enzymes and has been suggested to be essential modulator of ECM turnover (Villarreal et al., 2011; Luna et al., 2009). Due to the fact that miRNA-29 expression responded to different orientation of forces during OTM and it could be detected in GCF, it warrants investigating potential of this miRNA in GCF as a biomarker during OTM.
III. MATERIALS AND METHODS

A. Patient Selection

The research protocol was approved by the Institutional Review Board of University of Illinois (IRB# #2013-0183). Healthy subjects aged 10-17 years old were recruited from the patients receiving orthodontic treatment at the University of Illinois at Chicago’s (UIC) orthodontic clinic. The inclusion criteria included subjects requiring extraction of maxillary first premolars as part of their treatment with edgewise fixed appliances. The consent form was reviewed and obtained the eligible subjects and their parents (Appendix A). If the subjects were between ages of 11 and 15, they were also asked to sign the assent form (Appendix B).

To reduce the confounding effects, there were several exclusion criteria. They were as followed:

- subjects with impacted canines
- subjects with systemic diseases
- subjects exhibiting acute marginal gingivitis
- subjects with bleeding on probing in the areas of GCF collection
- subjects with periodontitis or with periodontal pockets of depth of 4mm or higher in areas of GCF collection.
- subjects with active caries
- subjects using any form of NSAIDS or bisphosphonates
B. **Collection of Gingival Crevicular Fluid**

Gingival crevicular fluid (GCF) was collected using absorbent Periopaper strips (OraFlow, Smithtown, NY, USA) inserted 1-2 mm into the gingival sulci at the distobuccal and mesiobuccal gingival margins of the canines (Figure 2). Before the collection, the canines were gently air-dried, any residual supragingival plaque was removed and the area of interest was isolated using cotton rolls. Periopapers were inserted at each site up to 4 times, for 60 seconds each, to ensure collection of an adequate amount of GCF (Griffiths, 2003). Samples were collected at six time points (Figure 3):

- **T0:** prior to bonding the fixed orthodontic appliances.
- **T1:** on the day of canine retraction, immediately prior to engaging the powerchains (American Orthodontics, Sheboygan, WI) onto the canine bracket. The powerchain for all the subjects was placed with the force of approximately 250g as measured by the Dontrix Force Gauge (Orthopli Corp, Philadelphia, PA).
- **T2:** 60 minutes after activating the power chain
- **T3:** 1 day after the canine retraction visit
- **T4:** 7 days after the canine retraction visit
- **T5:** between 5 weeks after initiation of canine retraction
The GCF samples with any blood, saliva or plaque contamination were discarded. After the collection, the GCF samples were stored in 1.5 ml Eppendorf tubes containing 0.2ml of DNase/RNase free phosphate-buffered saline (PBS) at 4°C immediately after collection. The tubes with Periopaper in PBS were spinned at 12.5-rcf to separate the paper and supernatant.
The supernatant was drawn and placed into 1 L of Trizol solution. The samples from each time point were stored at -80°C until use.

C. **RNA Assay and PCR**

Total RNA isolation was performed using Trizol lysis reagent (Life Technologies, Gaithersburg, MD) and miRNeasy minikit (Qiagen, Valencia, CA) consisting of phase separation using chloroform and spin-column RNA purification by centrifuging the aqueous phase through the silica gel membrane within the columns (Appendix C). The concentration of total RNA isolated from GCF at each time point was quantified using Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), software v.3.3. Total RNA in the samples was diluted to 2 ng/mL to standardize the amount of miRNA template into each PCR reaction. Quantitative RT-PCR miRNA reaction was performed using Taqman miRNA reverse transcription kit and Taqman miRNA assay RT primers (Tables 1 and 2) (Applied Biosystems, Foster City, California).
TABLE I. LIST OF MICRORNA\(\text{s}\) IN THE STUDY.

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Accession(^{a})</th>
<th>Kit Number(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-29a-3p</td>
<td>MIMAT0000086</td>
<td>002112</td>
</tr>
<tr>
<td>hsa-miR-29b-3p</td>
<td>MIMAT0000100</td>
<td>000413</td>
</tr>
<tr>
<td>hsa-miR-29c-3p</td>
<td>MIMAT0000681</td>
<td>000587</td>
</tr>
<tr>
<td>hsa-miR-101</td>
<td>MIMAT0000099</td>
<td>002253</td>
</tr>
<tr>
<td>hsa-miR-21</td>
<td>MIMAT0000076</td>
<td>000397</td>
</tr>
<tr>
<td>hsa-let-7d</td>
<td>MIMAT0000065</td>
<td>0002283</td>
</tr>
<tr>
<td>hsa-let-7i</td>
<td>MIMAT0000415</td>
<td>002221</td>
</tr>
<tr>
<td>hsa-let-7g</td>
<td>MIMAT0000414</td>
<td>002282</td>
</tr>
</tbody>
</table>

\(^{a}\) Based on Ver. 21 miRBase  
\(^{b}\) Based on Applied Biosystem Company
TABLE II. PRIMER SEQUENCES FOR qPCR.

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Primer Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-29a-3p</td>
<td>UAGCACCAUCUGAAUUCGGUUA</td>
</tr>
<tr>
<td>hsa-miR-29b-3p</td>
<td>UAGCACCAUUGAAUCAGUGUU</td>
</tr>
<tr>
<td>hsa-miR-29c-3p</td>
<td>UAGCACCAUUGAAUCGGUUA</td>
</tr>
<tr>
<td>hsa-miR-101-3p</td>
<td>UACAGUACUGUGAUACUUGAA</td>
</tr>
<tr>
<td>hsa-miR-21-5p</td>
<td>UAGCUUAUCAGACUGAUUGUUGA</td>
</tr>
<tr>
<td>hsa-let-7d</td>
<td>AGAGGUAGUAGGGUUGCAUAGUU</td>
</tr>
<tr>
<td>hsa-let-7i</td>
<td>UGAGGUAGUAGUUGUGCUGUU</td>
</tr>
<tr>
<td>hsa-let-7g</td>
<td>UGAGGUAGUAGUUGUACAGUU</td>
</tr>
</tbody>
</table>

<sup>a</sup>based on Applied Biosystems company website

The PCR reactions were performed in 96-well reaction PCR plates using a 7900HT Fast Real-Time PCR system (Applied Biosystems) and SDS 2.3 Sequencing Software (Applied Biosystems).

PCR Reactions were completed in 20 μL volumes consisting of:

1.33 μL of extracted miRNA from GCF
1 μL miRNA primer (Table 2)
10 μL of Taqman Universal PCR Master Mix (Applied Biosystems)
7.67 μL of PCR-grade water

The miRNAs were quantified in triplicates for each time point to reduce technical errors in preparation of PCR plate. The relative microRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen, 2001). Let7-d, Let7-g and Let7-i miRNAs were used as internal controls and relative proportion of each miRNA was calculated relative to the mean of the three reference miRNAs (Chen et al., 2013).

D. **Statistical Analysis**

The distribution of obtained data was investigated using Shapiro-Wilk test. Descriptive statistical analysis was reported as mean ± standard deviation. Data analysis were performed using IBM SPSS Statistics for Windows, V. 22.0, (Armonk, NY, IBM). Statistical significance was set at 0.05.
Figure 4. Methods flowchart
IV. RESULTS

We evaluated the changes in the temporal expression patterns of 5 different miRNAs during orthodontic tooth movement. The total of 70 subjects were recruited in the study. Hygiene deterioration and the subjects’ inability to be present for GCF collection at the required time points were major reasons for subject loss. At the end, 11 subjects with excellent oral hygiene during the study were enrolled in the study (3 males and 8 females) between the ages 10 and 18 (mean 14.5 years old). The distribution of subjects by gender were 3 males and 8 females and by race were 9 Hispanics, 1 African American and 1 Caucasian.

Shapiro-Wilk test showed non-normal distribution of the majority of studied miRNAs. To determine the significant changes in expression of each miRNA between time points of the study, non-parametric Wilcoxon signed-ranked test was used. Although the distribution was not normal, the descriptive statistics were also reported.

In all the gene groups, the change in expression of miRNA from T1 to T5 shows statistical significance consistently (p-value ranging from 0.005 to 0.047). Changes between T1 and T2 were only observed in miRNA-29b. Changes between T1 and T4 were observed in both miRNA-101 and miRNA-29b.
A. **Expression Patterns of each miRNA Between the Time points. (Figure 5)**

1. **miRNA-29a expression pattern**

   The expression of miRNA-29a was increased at 1 hr. then leveled down at 24 hr. after retraction. The expression increased gradually at day 7 and peaked up 5 weeks after retraction. There was a 1.6-fold increase in expression of miRNA-29a between T1 and its peak expression at T5, that was statistically significant (p-value=0.005). No significant difference was found any other time point (Figure 6).

2. **miRNA-29b expression pattern**

   The expression of miRNA-29b was increased at 1 hr then leveled down at 24 hr after retraction. The expression steeply increased at day7 then plateaued at 5 weeks after retraction. There was a 0.48-fold increase in expression of miRNA-29b between T1 and T2, 1.43-fold increase in expression between T1 and T4, and 1.6-fold increase between T1 and T5, time point with the peak expression levels of miRNA-29b. Statistically significant differences as: (p<0.05, p-value=0.005, p-value=0.009 and p-value=0.005 respectively), (Figure 7).

3. **miRNA-29c expression pattern**

   The expression of miRNA-29c was similar with those of miRNA-29a. There was a 1.45-fold increase in expression of miRNA-29a between T1 and its peak expression level at T5 (p<0.05, p-value=0.005). No significant difference was found between any other time points (Figure 8).
4. miRNA-101 expression pattern

The expression of miRNA-101 was similar to those of miRNA-29b; however, the significant difference was found only at day 7 and week 5 after retraction. was a 1.38-fold increase in expression levels of miRNA-101 between T1 and T4, and a 1.68-fold increase between T1 and T5 (p<0.05, p-value = 0.005 and p-value = 0.009 respectively). The changes between the remaining time points did not show statistical significance (Figure 9).

5. miRNA-21 expression pattern

The expression of miRNA-21 was plateaued until 7 days after retraction then increased to peak at week 5 after retraction. There was a 5.7-fold increase in expression of miRNA-21 between T1 and its peak expression level at T5 (p<0.05, p-value = 0.047). No significant difference was found between any other time points (Figure 10).

B. Comparison of Expression Profile of miRNA-29 Family

Analyzing all the time points together, Kruskal-Wallis test showed no statistical significant difference between miRNA-29a, -29b and -29c, p>0.05 (p-value ranging from 0.201 to 0.802), demonstrating a homogenous expression pattern of the miRNAs from the miRNA-29 family.

C. Relative Change between each Time point among the miRNAs

Comparison of responses between time points among the miRNAs, using Kruskal-Wallis detected statistically significant differences in ΔT1-T2 (p-value= 0.012). Mann-Whitney U post-hoc test showed the observed differences were between miRNA-21 and miRNA-101, -29a and -29b, (p-value = 0.004, p-value = 0.003 and p-value = 0.019 respectively). The detected
difference in expression profile of miRNA-21 shows variation in expression among the genes, suggesting a reduced probability of technical error.

**TABLE III. FOLD INCREASE IN EXPRESSION OF MI-RNAS BETWEEN TIME POINTS.**

<table>
<thead>
<tr>
<th></th>
<th>ΔT1-T2</th>
<th>ΔT1-T3</th>
<th>ΔT1-T4</th>
<th>ΔT1-T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold change</td>
<td>SE</td>
<td>Fold change</td>
<td>SE</td>
<td>Fold change</td>
</tr>
<tr>
<td>29a</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>29b</td>
<td>0.48</td>
<td>0.12</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>29c</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>101</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>-</td>
</tr>
</tbody>
</table>

*statistical significance set at α=0.05

Figure 5. Change in expression of miRNA-29a, -29b, -29c, -101 and -21 between time points.
Figure 6. Change in expression of miRNA-29a between time points.

Figure 7. Change in expression of miRNA-29b between time points.
Figure 8. Change in expression of miRNA-29c between time points.

Figure 9. Change in expression of miRNA-101 between time points.
Figure 10. Change in expression of miRNA-21 between time points.
V. DISCUSSION

MicroRNAs influence a significant, if not all, physiological pathways and their deregulation has been implicated in initiation and progression of various diseases (Borel et al., 2012). Such findings have created a novel field in medicine, at times referred to as “noncoding RNA revolution” to explore the use of miRNA as a diagnostic and therapeutic tool. Investigators have been able to associate clinically relevant circulatory miRNA expression profiles to various types of malignancies (Sun et al., 2014). Furthermore, miRNA mimicks and antagonists have been delivered locally and systemically, and successfully altered target gene expression with potential clinical implications (Borel et al., 2012; Cech and Steitz, 2014).

Many insidious diseases are diagnosed at late stage with poor prognosis due to inadequacy in available modalities to detect the disease at early stage. Early and effective diagnostic tools will prevent disease to progress to advanced stages. Recently secretory miRNAs have been in focus as an effective diagnostic tool for many diseases and pathologies. Cellular miRNAs are secreted into circulation and can be detected in different body fluids. Serum and plasma’s accessibility makes them an attractive medium for detecting biomarkers such as miRNA to screen for early onset of disease (Kosaka et al., 2010). In orthodontics, the treatment time varied among patients and it is sometimes difficult to determine treatment time or predict the treatment result in a certain period of time. If we had an effective tool to help predict the treatment time, the result would be more satisfaction.

Our study was the first to explore the profile change of certain secretory miRNAs during orthodontic tooth movement (OTM) in human. The objective of the present study was to evaluate some of epigenetic mechanisms, specifically the potential role of secretory miRNAs,
involved in tooth movement in human. In Dr. Atsawasuwan’s laboratory, we discovered the presence of secretory miRNAs in gingival crevicular fluid (GCF) (unpublished data). GCF samples were collected from two healthy volunteers, and subsequent gel electrophoresis in bioanalyzer showed presence of small RNAs at the size consistent with miRNAs in GCF. Studies have evaluated changes in GCF components and have linked the changes of tooth movement with expression patterns of biomarkers during OTM. GCF volume has also been investigated as a potential diagnostic tool for OTM, but it was found that the change in GCF volume is not a reliable indicator of OTM (Perinetti et al., 2013). Until now, there is no published instance of secretory miRNA detection in GCF. In this study we confirm the preliminary finding in our laboratory and further investigate in the response of certain miRNAs to the directional force during OTM.

GCF could be collected by different methods i.e. absorbent paper strip, paper points, and capillary tubes (Guentsch et al., 2012). Literature showed that method of GCF collection influenced the volume and the quality of GCF samples (Griffiths, 2003). Here we collected GCF sample using periopaper strips. This technic has been reported to be the method of choice for studying biomarkers in GCF due to its non-invasiveness and simplicity (Guentsch et al., 2012).

MiRNA-101, miRNA-21 and miRNA-29 family (a/b/c) were selected based on their potential involvement in molecular and cellular pathways associated with OTM. Recent studies have shown that miRNAs-29a/b/c, and miRNA-21 were involved in regulation osteoblasts and osteoclasts and could influence expression of key tissue remodeling agents such as TNFα. Kagiya and Nakamura (2013) found an increase in miRNA-29b and miRNA-21 during osteoclast differentiation in TNF-α/ RANKL-treated cells, suggesting the two miRNAs play a role in in TNFα -regulated osteoclast differentiation. Sugatani et al. (2011) demonstrated up-regulation of
miRNA-21 in RANKL-induced osteoclastogenesis and reported impaired osteoclastogenesis resulted by miRNA-21 silencing. They proposed a positive feedback model involving miRNA-21, programmed cell death 4 (PDCD4) protein, and c-Fos, a significant transcription factor for osteoclastogenesis.

Li et al. (2012) investigated contribution of miRNAs in post-transcriptional control of periodontal ligament-associated protein-1 (PLAP-1), a regulator of osteogenic differentiation of PDLCs, by analyzing miRNA expression profiles of PDLCs at various stages of osteoblast differentiation. They reported that miRNA-21 and miRNA-101 directly decreased expression of PLAP-1 during differentiation. MiRNA-101 has also been found to be protective against fibrogenesis through modulation of transforming growth factor beta (TGF-β) pathway in cardiac fibroblasts and hepatocytes (Zhao et al, 2015; Tu et al., 2014). TGF-β superfamily of growth factors are expressed by PDLCs and osteoblasts, and are broadly involved in regulation of matrix production and bone formation. The potential influence of MiRNA-101 on TGF-β, found in PDLCs, in addition to its regulatory role on PLAP-1, deemed it a noteworthy miRNA to investigate. We found a significant increase in expression of miRNA-101, 7 days after initial application of orthodontic force, and a subsequent increase leading to its peak levels at 5-week time point. MiRNA-101 modulates TGF-β, and PLAP-1, both involved in anabolic processes, which could explain its increase in later time points. Even though miRNA-21 did peak at 5-week time point similar to the other miRNAs in the study, it did show variation in overall expression pattern as compared to miRNA-101, miRNA-29a and miRNA-29b.

Franceschetti et al. (2013) investigated roles of miRNA-29 in osteoclastogenesis using murine macrophage. The authors found an increase in expression of all three members of miRNA-29 family during osteoclastogenesis, and repression of osteoclastogenesis process by
inhibition of miRNA-29. The finding led the authors to extrapolate that miRNA-29 promoted osteoclastogenesis.

MiRNA-29 is also involved in regulation of osteoblast differentiation by modulating Wnt gene pathway. Wnt pathway supports osteogenesis by positive regulation of osteoblasts and OPG and concurrently inhibiting RANKL (Del Carpio-Cano et al., 2013). Kapinas et al. (2010) showed that miRNA-29a promoted osteoblast differentiation in primary human osteoblast cell cultures by suppressing Wnt pathway inhibitors. A study by Li et al. (2009) showed miRNA-29b supported osteoblast differentiation by targeting inhibitors of osteoblastogenesis, and modulated ECM protein accumulation during mineralization stage by preventing protein accumulation and allowing for mineral deposition. They reported that miRNA-29b reached its peak levels on day 28 during the mineral deposition period. Kapinas et al. (2009) also presented evidence of down-regulation of another non-collagenous matrix protein, osteonectin, by miRNA-29 family. The study showed increased osteonectin protein levels in osteoblastic cells in the presence of miRNA-29 inhibitors, whereas decreased osteonectin was exhibited in the presence of miRNA-29 precursors. The expression levels of miRNA-29a and miRNA-29c, were both increased during osteoblastic differentiation. The authors also showed that miRNA-29b decreased multiple inhibitors of osteoblast differentiation, such as HDAC4, transforming growth factor-beta (TGF-β), activin receptor IIA, β-catenin, and dual specificity phosphatase 2.

Chen et al. (2015) studied changes in expression of miRNA-29 and their target extracellular matrix (ECM) genes in periodontal ligament cells (PDLCs) in response to compression and tension forces. Under compressive forces, up-regulation of expression in miRNA-29a,b and c was observed, with the highest increase in relative expression of miRNA-29b. Furthermore, the target ECM genes in PDLCs were down-regulated in presence of miRNA-
29b mimics but demonstrated an increase in their expression in presence of miRNA-29b inhibitors.

Placement of reciprocal force, as was done in this study, causes tipping of the canine and creating areas of both resorption and apposition in the surrounding periodontium. Considering involvement of miRNA-29 in both osteoblastic and osteoclastic activity as demonstrated by the literature, we expected to find an increase in expression of the miRNA-29 family at the collected sites. Although we detected an immediate up-regulation of three members of miRNA-29 family at 1 hour after application of directional force, only the change in miRNA-29b was statistically significant. The finding is consistent with a supporting role of miRNA-29 in osteoclastogenesis. Upon orthodontic force activation, osteoclast recruitment and osteoclastic activity has a fairly rapid response to orthodontic force. A significant increase in RANKL and decrease in OPG levels in GCF was reported as early as 24 hours after initiation of force (Nishijima et al., 2006). The rapid early increase of the miRNA-29 expression could be an event to promote osteoclastogenesis during tooth movement. In addition, miRNA-29b upregulation has been reported during the mineralization stage of bone remodeling (Li et al., 2009). In this study we observed late increase of miRNA-29b expression at 5-week time point. The observed expression pattern of miRNA-29 could be due to the potential role of miRNA-29b on the anabolic process during OTM. Although this study is a preliminary study to investigate the expression profile change, it provides information that could lead to more insight into how miRNA plays roles during OTM.

The course of orthodontic therapy is influenced by many factors including the appliances, patient compliance and the individual’s biological response to the orthodontic force. Medicine has recognized patients’ variations in response to therapy and is evolving into
personalizing the therapy to the individual’s biology. Similarly, to improve patients’ treatment outcome, orthodontics will most likely adopt the principle of personalized therapy by supplementing the mechanotherapy with interventions that are appropriate for individual’s biology and clarifying the molecular and cellular biology of OTM is a critical step to achieve that goal.

A. Limitations of the Study

Adults were not recruited into the study due to evidence of differences in GCF composition in adults vs. adolescents, which could lead to inconsistencies if the data from the two groups were combined (Kawasaki et al., 2006). Therefore, the findings of the study can not be generalized to individuals of all age groups. Due to the demographics of the patient population and location of the UIC orthodontic clinic, the racial distribution of subjects was skewed. Hispanic females made up the majority of subjects. We only report the approximate initial force but elastomeric chains are subject to decay in force magnitude with time (Eliades et al., 2004). Another variable that could be followed in future studies is to measure the amount of movement of the teeth subjected to the force to correlate amount of movement to the change in expression.

Orthodontic appliances establish an area for accumulation of plaque and local inflammation, consequently influencing the acquired GCF samples. Putting the subjects on a proper oral hygiene regimen could prevent loss of subjects due to inadequate oral hygiene and reduce the confounding impact of inflammation caused by the plaque. However, in this study the subjects exhibited excellent oral hygiene and showed no gingival inflammation during the study.
Despite the exploratory nature of the study, we aimed to recruit all the patients that met the inclusion criteria in our clinic. Seventy subjects were initially enrolled but we faced a high rate of subject loss primarily due to elimination of the subjects whose Periodent showed any visible signs of contamination, and the stringent timetable for sample collection not allowing any flexibility in scheduling the appointments.

B. **Normalization to Reference Gene**

Normalization is crucial step in determining changes in expression of miRNA between samples and its purpose is to remove variation between samples and isolating the change due to treatment effect (Sanders et al., 2014). There are traditionally used housekeeping genes that are used in quantitative studies of miRNA, depending on the source medium of the miRNA. Since there were no previous comparative studies that analyzed miRNA in GCF, a serum derivative, we selected a group of miRNAs (Let-7d, Let-7g, Let-7i) that had shown stable expression level in various serum samples and were reported by Chen et al. (2013) to be suitable options to be used as internal control for serum miRNA normalization.
VI. CONCLUSION

This was the first study to explore the in vivo response of secretory miRNAs during orthodontic tooth movement. The presence of miRNAs in GCF was evident. Furthermore, our findings indicated that application of orthodontic force caused alterations in the expression of secretory miRNA in GCF, with miRNA-29b demonstrating the most significant temporal changes among the studied miRNAs in the duration of the study. All three miRNAs of miRNA29 family showed a homogenous expression pattern. The peak levels of expression were found to be at the 5-week time point after application of a directional force.

Considering the novelty of subject of the study and lack of prior comparable studies, we considered this a preliminary study that requires validation by further investigation such as increased sample size, grouping subjects by age, race and gender. Parallel protein profile during the study will elucidate more insight into the role of miRNAs during tooth movement. Further studies on miRNAs are needed to elucidate additional dynamics and signaling pathways of biological processes that occur during OTM. The findings can also translate to clinical diagnosis and therapeutics modalities in different conditions involving bone remodeling.
CITED LITERATURE


Uematsu, S., Mogi, M., Deguchi, T.: Interleukin (IL)-1 beta, IL-6, tumor necrosis factor-alpha, epidermal growth factor, and beta 2-microglobulin levels are elevated in gingival
crevicular fluid during human orthodontic tooth movement. J. Dent. Res. 75(1);562-567:1996.


Appendix A

PATIENT CONSENT FORM

University of Illinois at Chicago
Consent/Parental permission and Authorization for Participation in Biomedical Research

Gingival crevicular fluid characterization during orthodontic treatment

If you are a parent or legal guardian of a child who may take part in this study, permission from you is required. The assent (agreement) of your child is also required. When we say "you" in this consent form, we mean you or your child; "we" means the research team.

Why am I being asked?

You are being asked to participate in a research study. Researchers are required to provide a consent form such as this one to tell you about the research, to explain that taking part is voluntary, to describe the risks and benefits of participation, and to help you to make an informed decision. You should feel free to ask the researchers any questions you may have.

Principal Investigator Name and Title: Assistant Professor Phimon Atsawasuwan
Department: Orthodontics
Address and Contact Information: Rm #247, College of Dentistry, University of Illinois at Chicago
Emergency Contact Name and Information: Dr. Phimon Atsawasuwan Tel: 312-355-4798
Sponsor: College of Dentistry, University of Illinois at Chicago

You are being asked to be a subject in a research study about “Gingival crevicular fluid characterization during orthodontic treatment” conducted by Assistant Professor Phimon Atsawasuwan, Department of Orthodontics, College of Dentistry at the University of Illinois at Chicago (UIC). The purpose of this study is to understand the composition of gingival crevicular fluid (liquid that stays between cervical tooth/root surface and gum at the gum line) when the wire moves teeth during the braces. We will collect gingival crevicular fluid and analyze to identify the unique protein that is related to tooth movement.

You have been asked to participate in the research because you have scheduled an appointment for the orthodontic tooth movement and may be eligible to participate in this study. We ask that you read this form and ask any questions you may have before agreeing to be involved in the research project.

Your participation in this research is voluntary. Your decision whether or not to participate will not affect your current or future dealings with the University of Illinois at Chicago. If you decide to participate, you are free to withdraw at any time without affecting that relationship.

Short Study Title: Gingival crevicular fluid characterization during orthodontic treatment
Version #4, date April, 12, 2013
Approximately 50 subjects may be involved in this research at UIC.

**What is the purpose of this research?**

This research is being done to better understand the composition of gingival crevicular fluid (liquid that stays between cervical tooth/root surface and gum at the gum line) due to the wire moves teeth during the braces. The composition of the proteins and changes in the gingival crevicular fluid may be an indicator for the development of gum disease.

**What procedures are involved?**

The crevicular fluid collection will be performed at Orthodontic clinic RM#131 and the analysis of the sample for this research will be performed at Rm #247, College of dentistry, University of Illinois at Chicago. The fluid will be collected two times, a month apart

The collection of fluid will be performed after your teeth are cleaned and absorbent paper strips will be inserted gently in the space between your gum and tooth surface around the gum line and let the paper absorb the fluid. Then the paper will be removed and kept in a storage solution for further analysis.

If you are taking pain medication for post-operative orthodontic treatment, you will only be allowed to take acetaminophen and not ibuprofen or aspirin. This restriction on pain medication would only apply to the study visits when samples are collected.

If you agree to be involved in this research project, we would ask you to let one of researcher in our team collecting your crevicular fluid, your name, medical number and date of birth for research purposes. The information will be coded with a study ID number to protect confidentiality and kept in a locked cabinet.

**What are the potential risks and discomforts?**

You may experience some mild discomfort from the sample collection, which includes the removal of plaque from the around the gum line. There is also a small risk of loss of confidentiality; others outside the research may become aware you are participating in the research. However, the researchers will take all steps to protect your confidentiality

**Are there benefits to taking part in the research?**

This study is not being done to improve your condition or health. You will receive no direct benefit from your participation in this research; however, the knowledge gained from this research may help dentists and orthodontist develop ways to prevent complications from braces.

**What other options are there?**

You have the option to not participate in this study. A decision not to participate will in no way affect the patient’s dental procedure or care.
What about privacy and confidentiality?

The people who will know that you are a research subject are members of the research team, and if appropriate, your physicians and nurses. No information about you, or provided by you, during the research, will be disclosed to others without your written permission, except if necessary to protect your rights or welfare (for example, if you are injured and need emergency care or when the UIC Office for the Protection of Research Subjects monitors the research or consent process) or if required by law.

Study information which identifies you and the consent form signed by you will be looked at and/or copied for examining the research by:

- UIC Office for the Protection of Research Subjects, State of Illinois Auditors

When the results of the research are published or discussed in conferences, no information will be included that would reveal your identity.

By signing this form, you are also authorizing the researchers to use the health information provided by you for the purposes of this research. This includes the information as described within this form and specifically includes name, date of birth, and medical record number.

This authorization does not have an expiration, but you may withdraw your permission for the use of your health information for this study at any time by sending a letter to the following person: Dr. Phimon Atsawasuwan, University of Illinois at Chicago (UIC), College of Dentistry, 801 S Paulina St, Room #247 Chicago, IL 60612. If you cancel this authorization, the investigators may still use and disclose the health information they have already obtained as necessary to maintain the integrity and reliability of research.

What are the costs for participating in this research?

There is no additional cost to you for participating in this research.

Will I be reimbursed for any of my expenses or paid for my participation in this research?

There is no reimbursement or payment for participating in this research.

Can I withdraw or be removed from the study?

If you decide to participate, you are free to withdraw your consent and discontinue participation at any time without affecting your future care at UIC.

Who should I contact if I have questions?

Contact the researchers Assistant Professor Phimon Atsawasuwan at 312-355-4798:

- if you have any questions about this study or your part in it,
- if you have questions, concerns or complaints about the research.
What are my rights as a research subject?

If you have questions about your rights as a research subject or concerns, complaints, or to offer input you may call the Office for the Protection of Research Subjects (OPRS) at 312-996-1711 or 1-866-789-6215 (toll-free) or e-mail OPRS at uicirb@uic.edu.

Remember:

Your participation in this research is voluntary. Your decision whether or not to participate will not affect your current or future relations with the University. If you decide to participate, you are free to withdraw at any time without affecting that relationship.

Right to Refuse to Sign this Authorization

You do not have to sign this Consent/Authorization. However, because your health information is required for research participation, you cannot be in this research study if you do not sign this form. If you decide not to sign this Consent/Authorization form, it will only mean you cannot take part in this research. Not signing this form will not affect your treatment, payment or enrollment in any health plans or your eligibility for other medical benefits.

Signature of Subject or Legally Authorized Representative

I have read (or someone has read to me) the above information. I have been given an opportunity to ask questions and my questions have been answered to my satisfaction. I agree to participate or give permission for my child to participate in the research study and authorize the researcher to use and share my/my child’s health information for the research. I will be given a copy of this signed and dated form.

__________________________________________  _________________________________
Signature of child (16-17 years old)            Date

__________________________________________  _________________________________
Signature (18 years and older)                  Date

__________________________________________  _________________________________
Printed Name (18 years and older)               Date

__________________________________________  _________________________________
Signature [parent(s)/legal guardian(s) if patient is under 18] Date

__________________________________________  _________________________________
Printed Name [parent(s)/legal guardian(s) if patient is under 18] Date

__________________________________________  _________________________________
Signature of Person Obtaining Consent           Date (must be same as subject’s)

Short Study Title: Gingival crevicular fluid characterization during orthodontic treatment
Version #4, date April, 12, 2013
Appendix B

PATIENT ASSENT FORM

University of Illinois at Chicago

ASSENT TO PARTICIPATE IN RESEARCH (aged 11-15 years old)

1. My name is Dr. Phimon Atsawasuwann.

2. We are asking you to take part in a research study because we are trying to learn more about how the teeth move and how the gum and bone that wrap around the teeth react during the braces.

3. If you agree to be in this study, your teeth will be cleaned and the tiny paper strips will be put in places close to your gum to collect some liquid from your gum and teeth today and one more time next month when you come back to see your orthodontist.

4. During the procedure, you might feel mild discomfort feeling on your gum but nothing will be harmful.

5. From this research, you will help dentists gain new knowledge to improve braces treatment.

6. Please talk this over with your parents before you decide whether or not to participate. We will also ask your parents to give their permission for you to take part in this study. But even if your parents say “yes” you can still decide not to do this.

7. If you don’t want to be in this study, you don’t have to participate. Remember, being in this study is up to you and no one will be upset if you don’t want to participate or even if you change your mind later and want to stop.

8. You can ask any questions that you have about the study. If you have a question later that you didn’t think of now, you can call me 312-3554798 or ask me next time.

9. Signing your name at the bottom means that you agree to be in this study. Your doctors will continue to treat you whether or not you participate in this study. You and your parents will be given a copy of this form after you have signed it.

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<th>Name of Subject</th>
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<tr>
<td>Signature</td>
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Appendix C

MiRNeasy SERUM/PLASMA KIT PROTOCOL.

1. Prepare serum or plasma, or thaw frozen samples.

2. Add 5 volumes QIAzol Lysis Reagent to the sample (e.g., for 200 μl sample, add 1 ml QIAzol Lysis Reagent). Mix by vortexing or pipetting up and down.

3. Incubate the homogenate at room temperature (15–25°C) for 5 min.

4. Add 3.5 μl miRNeasy Serum/Plasma Spike-In Control (at 1.6 x 108 copies/μl).

5. Add chloroform of an equal volume to the starting sample and cap tube securely (e.g., for 200 μl sample, add 200 μl chloroform). Shake vigorously for 15 s.

6. Incubate at room temperature for 2–3 min.

7. Centrifuge for 15 min at 12,000 x g at 4°C.

8. Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transferring any interphase. Add 1.5 volumes of 100% ethanol (e.g., for 600 μl aqueous phase, add 900 μl ethanol). Mix thoroughly by pipetting.

9. Pipet up to 700 μl sample, including any precipitate, into an RNeasy MinElute spin column in a 2 ml collection tube. Close the lid and centrifuge at ≥8000 x g for 15 s at room temperature. Discard the flow-through.

10. Repeat step 9 using the remainder of the sample.

11. Add 700 μl Buffer RWT to the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.

12. Pipet 500 μl Buffer RPE onto the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.

13. Add 500 μl of 80% ethanol to the RNeasy MinElute spin column. Close the lid, and centrifuge for 2 min at ≥8000 x g. Discard the flow-through and the collection tube.

14. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column and centrifuge at full speed for 5 min to dry the membrane. Discard the flow-through and the collection tube.

15. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 μl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.
# VITA

**NAME:** Paul Lazari  

**EDUCATION:**  
DDS, New York University College of Dentistry, New York City, New York, 2013  
B.S., Genetics, University of California at Davis, Davis, California, 2007  

**RESEARCH EXPERIENCE:**  
University of Illinois at Chicago College of Dentistry, Department of Orthodontics, Atsawasuwan Lab (9/2013-2016)  
MicroRNAs as biomarkers for orthodontic tooth movement  
New York University College of Dentistry, Department of Orthodontics, (6/2009-2013)  
Examine course evaluation in terms of reliability, precision, and validity and pilot a new type of course evaluation form that examines the introduction of rubrics in evaluations. *(Posters presented at 2011 ADEA Conference in San Diego 2012 ADEA Conference in Orlando 2013 ADEA Conference in Seattle)*  
Utilized Statistical Package for Social Sciences (SPSS) Perception of San Juan residents in regards to Puerto Rico Birth Control Studies. *(Poster presented at IADR Conference, April 2009, Miami)*  
Examination of gene therapy in oral cancer by non-viral gene delivery vectors.  

**HONORS:**  
Pierre Fauchard Foundation award  
NYUCD Rahmsdorf Scholarship  
OKU Academic Excellence award  
American Association of Orthodontists award for pre-doctoral student  
American Association of Public Health Dentistry award
VITA (Continued)

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