Nitric Oxide Changes in Gingival Crevicular Fluid Following Orthodontic Force Application

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science (Orthodontics)

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Nitric oxide (NO) plays a role in regulating the rate of orthodontic tooth movement (OTM) in rat models; however, in humans this role remains less clear. In this study, samples of gingival crevicular fluid (GCF) were collected from each maxillary central incisor and first and second molar immediately before (T0), 1 hour after (T1), and 3-4 days after (T2) application of light orthodontic forces in thirteen male participants (ages 11-18 years) undergoing orthodontic therapy. NO levels were measured in each GCF sample, and significantly higher NO levels (p<0.05) were found at T1 at the buccal surfaces of the central incisors when compared to the posterior teeth. The results indicate a possible role for NO in OTM at the pressure sites of incisors at early time points. Further studies are required to determine whether NO levels in the PDL of human teeth are affected by the magnitude of an applied force.
ACKNOWLEDGEMENTS

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Manuscript in preparation:

**Nitric oxide changes in gingival crevicular fluid after orthodontic force application**

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<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>Core Binding Factor 1</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-Derived Relaxing Factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>GCF</td>
<td>Gingival Crevicular Fluid</td>
</tr>
<tr>
<td>GLMM</td>
<td>Generalized Linear Mixed Models</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LLLT</td>
<td>Low-level Laser Therapy</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-nitroarginine-methyl ester</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>L-$N^G$-monomethyl arginine</td>
</tr>
<tr>
<td>MGI</td>
<td>Modified Gingival Index</td>
</tr>
<tr>
<td>NED</td>
<td>$N$-1-naphylethlyenediamine dihydrochloride</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear Factor Kappa-beta</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OTM</td>
<td>Orthodontic Tooth Movement</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>PDL</td>
<td>Periodontal Ligament</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PI</td>
<td>Plaque Index</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid Hormone</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor Activator of NFκB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of NFκB ligand</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related Transcription Factor 2</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble Guanylyl Cyclase</td>
</tr>
<tr>
<td>T0</td>
<td>Sampling time point #1 (baseline), samples collected immediately prior to bonding with maxillary braces</td>
</tr>
<tr>
<td>T1</td>
<td>Sampling time point #2, samples collected one hour after insertion of maxillary archwire from 16-26</td>
</tr>
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<td>T2</td>
<td>Sampling time point #3, samples collected 3-4 days following bonding of maxillary teeth with braces and insertion of maxillary archwire</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-alpha</td>
</tr>
</tbody>
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CHAPTER 1. INTRODUCTION

1.1 Significance of Project

Reducing the duration of patient treatment is a primary goal for orthodontists (Norton and Burstone, 1989). The development of methods to increase the rate of orthodontic tooth movement (OTM) has been sought by clinicians as a way to shorten the amount of time a patient spends in braces. Since OTM is characterized by sequential reactions of the periodontal tissues and alveolar bone to biomechanical forces, factors influencing these processes may enhance the rate of OTM and are of particular interest to clinicians in the field of orthodontics (Krishnan and Davidovitch, 2006; Shirazi et al., 2002).

During the application of an orthodontic force, the alveolar bone on the pressure side undergoes successive cycles of bone resorption and formation, while the bone on the tension side predominantly undergoes continuous bone formation (Nilforoushan et al., 2002). Cellular factors that influence the above cycles may alter the rate of OTM, and thus advancing this body of knowledge is especially important in the field of orthodontics (Nilforoushan et al., 2002).

Nitric oxide, (NO), a short-lived, highly-reactive free radical, has been shown to play a role in the process of OTM. NO is a signalling molecule that is involved in the mediation of bone mechanical loading as well as in the regulation of bone function and turnover (Nilforoushan and Manolson, 2009). In rat models, elevated levels of NO have been shown to enhance the rate of OTM (Akin et al., 2004; Hayashi et al., 2002; Shirazi et al., 2002). The role of NO in OTM in humans, however, remains less understood.
Recent studies in OTM have used gingival crevicular fluid (GCF), a fluid that arises at the gingival margin, to analyze a variety of biomarkers involved in OTM (Krishnan and Davidovitch, 2006). Samples of GCF are commonly used because of their non-invasive nature and ease of sampling, as well as their diagnostic potential (Krishnan and Davidovitch, 2006; Tözüm et al., 2007). Previous studies on periodontal disease and implant stability have shown that NO is detectible in GCF (Genc et al., 2013; Güncü et al., 2008), and the use of this method in orthodontics can provide a considerable amount of knowledge toward the role of NO in OTM in humans.

The purpose of this study, therefore, is to investigate the changes in GCF levels of NO before and during the application of an orthodontic force.

1.2 Objective

The objective of this study was to measure the levels of NO in the GCF of specific teeth before and during the application of light continuous orthodontic forces.

1.3 Hypothesis

Nitric oxide levels are increased in the GCF of a tooth after an orthodontic force has been applied and sustained on that tooth, when compared to baseline and teeth to which an orthodontic force has not been applied.
2.1 Nitric Oxide

2.1.1 Introduction

NO is a free radical that acts as a signalling molecule regulating many physiological processes in the human body. NO was first identified as an endothelium-derived relaxing factor (EDRF) that affected vascular smooth muscles resulting in relaxation of these muscles and subsequent vasodilation by Furchgott and Zawadzki in 1980. Later that decade, EDRF, which is released from arteries and veins, was identified to be NO (Ignarro et al., 1987; Palmer et al., 1987). NO was shown to be synthesized from the amino acid L-arginine (Palmer et al., 1988) and research on NO steadily increased as it was found to have anti-tumor activities (Drapier et al., 1988) and immunoregulatory effects (Moilanen et al., 1997).

An earlier discovery that NO stimulates soluble guanylyl cyclase (sGC) causing vascular relaxation effects (Katsuki et al., 1977) contributed to the establishment of NO as a signaling molecule in the cardiovascular system. Years later in 1998, Pfizer utilized this knowledge in the development Sildenafil (Viagra®), which affects vasodilation through NO via sGC activation. This revolutionized the management of erectile dysfunction and resulted in an exceptional increase in NO popularity (Yetik-Anacak and Catravas, 2006). After the Nobel Prize in Physiology and Medicine was awarded that same year to the scientists who discovered the effects of NO in their laboratory (Furchgott, Ignarro and Murad), worldwide interest in NO research was extensively prevalent (Yetik-Anacak and Catravas, 2006).
The increased popularity and NO research that was then undertaken led to the realization that NO plays a variety of diverse roles in the body. NO has been implicated in the pathogenesis of diseases ranging from hypertension to septic shock and dementia (Moncada and Higgs, 1993). The number of diseases that are now associated with altered NO homeostasis are numerous, and terms such as “endothelial dysfunction” have become synonymous with reduced biological activity of NO (Catravas et al., 1983; Yetik-Anacak and Catravas, 2006).

Overall, the roles of NO include acting as a signaling molecule involved in dilation of blood vessels (Palmer et al., 1987), transmission of neural signals, cardiovascular homeostasis, immune response (Lamas et al., 1991; Lamas et al., 1992) and in the pathogenesis of countless diseases (Yetik-Anacak and Catravas, 2006). NO is also involved in bone remodeling induced by mechanical loading, and several investigators have reported that NO plays a role in OTM (Inami et al., 2009). The following sections will focus on NO production and associated enzymes, its modes of action, and particularly the role of NO in bone and OTM.

### 2.1.2 Biosynthesis of Nitric Oxide

NO is synthesized enzymatically from the amino acid L-arginine by one of three enzymes referred to as nitric oxide synthases (NOS) (Yetik-Anacak and Catravas, 2006). This is completed in a two-step process via the formation of N-hydroxyl L-arginine (Dawson and Snyder, 1994). Short lived NO binds to oxygen producing two stable end products, nitrate (NO$_3^-$) and nitrite (NO$_2^-$) yielding L-citrulline as a coproduct (Ignarro et al., 1993; van't Hof and Ralston, 2001). Figure 1 depicts the L-arginine-NO pathway, adapted from van't Hof and Ralston (2001).
Figure 1: L-arginine-NO pathway. NO is synthesized from L-arginine and oxygen by the NOS group of enzymes. NO is an extremely reactive molecule; once produced it reacts with oxygen to form nitrite and nitrate. (Adapted from van’t Hof and Ralston (2001)).

The reaction depicted in Figure 1 can be inhibited by substituted arginine analogues, such as L-N^G-monomethyl arginine (L-NMMA) and L-nitroarginine-methyl ester (L-NAME) (van’t Hof and Ralston, 2001). NO can also be generated pharmacologically by compounds such as organic nitrates (ie. nitro-glycerine) and sodium nitro-prusside, which are used clinically as vasodilators (Feelisch and Stamler, 1996). Additionally, NO can be generated nonenzymatically in the acidic conditions of the stomach (Benjamin et al., 1994).

NO binds to the heme moiety of the NOS enzyme, which induces a conformational change resulting in the inhibition of enzymatic activity. This therefore produces an autoregulatory feedback loop by which increased NO levels limit NO production (Rogers and Ignarro, 1992).

2.1.2.1 Nitric Oxide Synthases

Nitric oxide synthases (NOS) are a family of enzymes resembling cytochrome P-450. They act to oxidatively remove the terminal guanidine nitrogen from L-arginine to form
citrulline and NO (Moilanen and Vapaatalo, 1995); also see Figure 1. Three major types of NOS have been characterized: 1) Endothelial NOS (eNOS), 2) Neuronal NOS (nNOS), and 3) Inducible NOS (iNOS) (Yetik-Anacak and Catravas, 2006). eNOS and nNOS are referred to as constitutive isozymes, which are constitutively expressed and yield less NO with several physical and chemical stimuli, whereas the inducible isoform (iNOS) produces a larger amount of NO through de novo synthesis of the enzyme in response to proinflammatory cytokines or bacterial endotoxin (Hikiji et al., 1997). Each isoform was originally cloned from different cell types, and these enzymes are now known to be expressed in several tissues. Table 1 summarizes these cells and tissue expression types (Guzik et al., 2003).

Table 1: Cell derivations and tissue expression of the three NOS isoforms (adapted from Guzik et al. (2003))

<table>
<thead>
<tr>
<th>Originally cloned from</th>
<th>eNOS</th>
<th>nNOS</th>
<th>iNOS</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Endothelial cells</td>
<td>Neuronal cells</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Tissue expression</td>
<td>Cardiac myocytes</td>
<td>Skeletal muscle</td>
<td>Cardiac myocytes</td>
</tr>
<tr>
<td></td>
<td>Platelets</td>
<td>Neutrophils</td>
<td>Glial cells</td>
</tr>
<tr>
<td></td>
<td>Neurones</td>
<td>Vascular smooth</td>
<td>Vascular smooth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>muscle cells</td>
<td>muscle cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endothelium</td>
<td>Neurones</td>
</tr>
</tbody>
</table>

2.1.3 Cellular Actions of NO

NO is a highly reactive molecule, and resultantly has numerous molecular targets. Iron appears to be a preferred receptor for NO; thus NO exerts many of its effects through binding to iron containing enzymes (Collin-Osdoby et al., 1995). In platelets and in vascular smooth muscle, NO binds to the heme moiety of sGC, which increases the activity of this enzyme.
The subsequent elevated levels of cGMP cause an activation of a cascade of phosphorylation events of cGMP dependent protein kinases, resulting in smooth muscle relaxation and inhibition of platelet adhesion (van't Hof and Ralston, 2001).

Additionally, NO reacts with sulphydryl residues and iron-sulphur centres in protein to produce other biological effects. The effect of iron-sulphur centres is reported to be responsible for the inhibition of cell growth due to high NO concentrations. This inhibition occurs through the interaction with mitochondrial aconitase, which is a critical enzyme in the Krebs cycle (Feelisch and Stamler, 1996). Another molecular target for NO has been shown to be GTPase p21\textsuperscript{ras}. NO nitrosylates the cysteine residue 118 (Cys118) of p21\textsuperscript{ras} which results in the activation of GTPase activity and subsequent stimulation of the NFκB and map kinase signal transduction pathways (Lander et al., 1995).

NO also plays a role in the inflammatory response of the body. NO reacts with superoxide anions or other oxygen-derived free radicals to form such highly reactive molecules as the hydroxyl radical or the peroxynitrite anion (Feelisch and Stamler, 1996). The production of these toxic moieties induces lipid peroxidation, contributing to the tissue damage associated with the inflammatory response (Lipton et al., 1993).

2.1.4 NO in Inflammation

During immune activation in response to inflammation, NO levels are frequently elevated. High levels of NO are associated with various inflammatory diseases and autoimmune disorders (Collin-Osdoby et al., 1995). As part of the inflammatory response, cytokines, tumor necrosis factor-α (TNF-α), and interleukin-1P are produced. This leads to the activation of
intracellular nuclear transcription factors such as NFκB within macrophages, leading to the upregulation and transcription of NOS. Ultimately, this results in the synthesis of NO in response to inflammatory conditions (Collin-Osdoby et al., 1995). A positive feedback loop appears to be present, since NFκB can also be activated by NO, thus NO production can be amplified during inflammation (Lowenstein et al., 1994). Large amounts of NO can be toxic and pro-inflammatory, however this amplification can be tempered by modulatory factors or other cytokines (TGF-P, IL-8, IL-10 etc.) (Schini et al., 1992).

2.1.5 Hormonal Modulation of NO Production

Hormones represent a class of widely circulating chemical factors that play a role in the production of NO, both in the short and long term. The rapid effects of hormones manifest as the phosphorylation of the eNOS isoform, whereas longer term effects are a result of alterations in the amount of the eNOS protein (Duckles and Miller, 2010). Hormones that increase NO production through these mechanisms include estrogen, progesterone, insulin, and growth hormone. Conversely, glucocorticoids, progesterone, and prolactin have been shown to decrease NO bioavailability through binding to repressor response elements on the eNOS gene and a variety of other mechanisms (Duckles and Miller, 2010). In osteoblasts and endothelial cells, estrogen stimulates eNOS activity and mRNA levels, leading to the hypothesis that NO derived from the eNOS pathway plays a role in mediating the effects of sex hormones in bone (van't Hof and Ralston, 2001).
2.2 NO and Bone

2.2.1 Introduction: Bone and Bone Cells

Bone is a complex tissue composed of several cell types, which work together to continuously undergo a process of renewal and repair known as ‘bone remodeling’ (van't Hof and Ralston, 2001). The bone cell types include osteoblasts, which form new bone, osteoclasts, which resorb bone, and osteocytes, which are believed to act as sensors of mechanical stress in the skeleton (van't Hof and Ralston, 2001). During the bone remodelling cycle, old or damaged bone is removed by osteoclasts through the secretion of proteolytic enzymes and acid onto the bone surface. Osteoclasts then undergo apoptosis and are replaced by osteoblasts which lay down new bone in the form of osteoid. Osteoid eventually calcifies and becomes mature bone. During this process, some osteoblasts become embedded in the bone matrix and become osteocytes (van't Hof and Ralston, 2001).

Osteoblasts are cells of mesenchymal origin which differentiate from bone marrow stromal cells upon the activation of the transcription factor Cbfa1 (MacDonald et al., 1987). Osteoclasts, alternatively, are multinucleated cells of hematopoietic origin which differentiate from precursors in the monocyte/macrophage lineage in response to co-ordinated expression of regulatory molecules such as RANK (receptor activator of NFκB), RANK ligand (RANKL), osteoprotegerin (OPG), and others (Teitelbaum, 2000). Finally, osteocytes are interconnected to each other and to cells on the bone surface through a series of long cytoplasmic processes that run through cannaliculi in the bone matrix. It is through this network that osteocytes detect and respond to changes in fluid flow thereby acting as sensors of mechanical stress (van't Hof and Ralston, 2001).
The regulation of bone remodelling is carried out by a variety of factors, including systemic hormones such as parathyroid hormone (PTH), 1,25 dihydroxyvitamin D3, sex hormones, and calcitonin, as well as by local factors such as NO, cytokines, growth factors, and prostaglandins (van't Hof and Ralston, 2001). The current belief is that many of these listed factors which regulate bone remodeling play a role in influencing the expression of RANK, RANKL, and OPG, forming a paracrine system that regulates osteoclast differentiation and function (Roux and Orcel, 2000; Teitelbaum, 2000).

2.2.2 NO in Bone

NO exerts pronounced effects on skeletal physiology and its interactions by a variety of factors have an effect on the communication pathways controlling bone cell mechanisms and remodeling. It has been shown that NO plays a role in bone cell function and bone turnover (Michell et al., 1999). eNOS has been shown to be expressed constitutively in bone marrow stromal cells, osteoblasts, osteocytes, and osteoclasts (Brandi et al., 1995; Helfrich et al., 1997; MacPherson et al., 1999). iNOS does not appear to be constitutively expressed in normal adult bone, although it has been observed in fetal bone which suggests a role in skeletal development (Helfrich et al., 1997; Hukkanen et al., 1999). Finally, nNOS, although previously reported to not be detectable in bone, has been shown to have evidence of nNOS protein expression in bone lining cells and in osteocytes (Fox and Chow, 1998).

Knockout (KO) model studies have given great insight into the role of the NOS enzymes. eNOS KO mice have shown reduced bone formation rates, bone mineral density, as well as reduced bone volume. Defects in osteoblast maturation and activity, as well as reduced number of osteoblasts were also seen (Aguirre et al., 2001). iNOS KO mice did not show any particular bone abnormalities under normal conditions (Cuzzocrea et al., 2003), and nNOS KO mice
demonstrated an increase in bone mineral density and a decrease in bone turnover (van't Hof et al., 2004).

2.2.2.1 The Effects of NO on Osteoclastic Bone Resorption

NO has been shown to have biphasic effects with regard to the role of NO in osteoclastic bone resorption. Low concentrations of NO have been shown to potentiate IL-1 induced bone resorption (Ralston et al., 1995), whereas high concentrations of NO have been shown to inhibit osteoclastic formation and activity (Nilforoushan et al., 2009; van't Hof and Ralston, 2001). Constitutive production of NO within osteoclasts has been suggested to be essential for osteoclast function, based on experimentation where NOS inhibitors were shown to inhibit the activity and motility of isolated osteoclasts (Brandi et al., 1995). iNOS and eNOS isoforms do not appear to be critical for osteoclast formation, however, as demonstrated through experimentation with animals with eNOS and iNOS deficiencies that did not show major defects in bone resorption under physiological conditions (van't Hof and Ralston, 2001). Under non-physiological conditions, however, the iNOS pathways have been shown to play a critical role in cytokine and inflammation induced bone loss (van't Hof and Ralston, 2001). Disrupting the nNOS gene has been shown to decrease the number of osteoclasts (van't Hof et al., 2004), whereas the number of osteoclasts in similar experiments involving eNOS (Aguirre et al., 2001) and iNOS (Cuzzocrea et al., 2003) did not change. Overall, NO and each NOS isoform has been shown to play a role, either indirectly or directly, in osteoclastic bone resorption.

2.2.2.2 The Effects of NO on Osteoblastic Bone Formation

NO also appears to have biphasic-like effects on osteoblastic bone formation. Low levels of NO constitutively expressed by osteoblasts seem to act as an autocrine stimulator of osteoblast
growth, as shown by in vitro studies (Riancho et al., 1995). In eNOS knockout animals, major defects in bone formation and osteoblastic activity were noted both in vivo and in vitro, indicating an important role for eNOS in osteoblast differentiation and function (Aguirre et al., 2001). The inhibitory effects on osteoblastic growth and differentiation seen at high concentrations of NO seem to be partly due to pro-apoptotic effects of NO on osteoblasts, mediated partly by cGMP (Mancini et al., 2000; Mogi et al., 1999).

2.3 Orthodontic Tooth Movement

2.3.1 Introduction

OTM is influenced by a variety of factors that play a role in the overall success of orthodontic treatment. These factors include periodontal health, oral hygiene, and orthodontic forces (Cardaropoli and Gaveglio, 2007). Upon the application of orthodontic forces, tooth movement occurs through remodelling changes in the dental and periodontal tissues. The applied force causes the compression of the periodontal ligament (PDL) on one side, (the “compression” side) while the opposite side is “stretched” and is referred to as the tension side (Dolce et al., 2002).

Mechanical loading causes a variety of changes in the bone and periodontal tissues, altering the vascularity and blood flow which results in the local synthesis and release of a variety of molecules. These molecules include cytokines, growth factors, neurotransmitters, colony-stimulating factors, and arachidonic acid metabolites (Zainal Ariffin et al., 2011). The following section will outline key anatomic and functional components of the PDL, details regarding the compression and tension sides during OTM, and the role that NO plays in OTM.
2.3.2 The Periodontal Ligament

The PDL is a soft, specialized connective tissue consisting of a complex organization of collagenous fibre bundles, cellular elements, and tissue fluids. It is situated between the bone that forms the tooth socket wall, and the cementum covering the root of the tooth, with a width in the range of 0.15-0.38 mm (Nanci, 2003). The principal functions of the PDL are to support the teeth in their sockets and attach the teeth to bone, all while withstanding the considerable forces associated with mastication. Additionally, the PDL acts as a sensory receptor to enable the proper positioning of the jaws during normal function (Nanci, 2003).

The cellular elements of the PDL include connective tissue cells (fibroblasts, cementoblasts, and osteoblasts), epithelial cell rests of Malassez, defense cells, and cells of neurovascular origin. Fibroblasts, being the most prevalent of all the cells in the PDL, are the principal cell element (Nanci, 2003). Additionally, there is a component of ground substance and neurovascular elements within the PDL (Newman et al., 2002). Collagen is the most abundant element found within the PDL, making up 51% of a mouse molar PDL composition (Nanci, 2003). NOS enzymes have been found to exist in fibroblasts, blood vessels, and nerves of the PDL (Nathan, 1992).

The PDL provides active stabilization of the teeth against prolonged forces of light magnitude. This concept implies that there is a threshold, above which orthodontic forces result in tooth movement facilitated primarily by changes in the PDL and periodontium (alveolar bone, gingiva, dental pulp, in addition to the PDL) (Proffit et al., 2007; Zainal Ariffin et al., 2011).
2.3.3 Tissue and Cellular Changes during OTM

Several metabolic changes in the periodontal tissues take place upon application of mechanical stress, enabling tooth movement. One hour following orthodontic force application, the thickness of the PDL was observed to change, and more significant changes were seen at 6 hours (Nakamura et al., 2008).

The response of the PDL depends on the magnitude of the force that is applied. If the force is applied for less than one second, the PDL fluid acts as a “shock absorber” and is incompressible. The alveolar bone bends slightly, and a piezoelectric signal, which creates a flow of an electric current as electrons are displaced from one part of the crystal lattice (bone) to the other, is generated (Proffit et al., 2007). When the force is applied for one to two seconds, PDL fluid will be expressed, and the tooth will move within the PDL space. As the pressure is sustained, blood vessels in the PDL dilate on the tension side, and are partially compressed on the pressure side. Alterations in blood flow and oxygen tension within the PDL then ensue, stimulating the release of prostaglandins and cytokines within minutes of the initial onset of pressure. Within hours, important metabolic changes begin to occur. cAMP levels are notably elevated four hours after the initial force application (Yousefian et al., 1995), and the GCF levels of two potent bone resorbing mediators, prostaglandin E (PGE) and interleukin-1 beta (IL-1 β), also show a significant increase (Grieve et al., 1994). Prostaglandin secretion has been detected as early as 15 minutes after force application (Ngan et al., 1990). Many osteoblast-associated markers such as alkaline phosphatase (ALP), bone sialoprotein, runt-related transcription factor 2 (Runx2)/corebinding factor 1 (Cbfa1), and RANKL are also induced within the PDL, these within 24 hours of force application. Osteocalcin and type I Collagen were observed thereafter,
between 24 and 48 hours after force application (Oshiro et al., 2002; Pavlin et al., 2000; Shiotani et al., 2001; Yasuda et al., 1998).

After two days, actual tooth movement occurs as osteoclasts resorb bone on the pressure side and osteoblasts form new bone on the tension side. If the applied pressure is too heavy, blood vessels in the pressurized area will occlude causing cell death. The osteoblasts and osteoclasts that are required for tooth movement must then be chemo-attracted from a distant site, rather than from the PDL itself. Thus, before tooth movement can occur, the lamina dura on the pressure side must be resorbed, and tooth movement can then occur within 7-14 days. This process, where heavy forces are involved, is referred to as “undermining resorption” (Diaz, 1978; Masella and Meister, 2006; Proffit et al., 2007). With regard to clinical reality, a small amount of undermining resorption usually occurs during OTM because, although it is ideal to always use very light, continuous forces, this is not always possible and small sites of undermining resorption may occur to facilitate tooth movement (Proffit et al., 2007).

Most recently, it has been reported that osteocytes also play a critical role in osteoclastic bone resorption during OTM. Matsumoto et al. (2013) applied an orthodontic force to the incisors and first molars of transgenic mice in which osteocytes were specifically ablated. They found that the amount of tooth movement that occurred in these mice after 12 days was significantly less than the amount of tooth movement that occurred in control mice. These results suggest that osteocytes also play an important role in OTM (Matsumoto et al., 2013).

2.3.4 NO in OTM

NO has been shown to be involved in OTM in rat models. When a general inhibitor of NOS activity, L-nitroarginine-methyl ester (L-NAME), and a NOS precursor, L-arginine, were
locally administered to the upper first molars or upper incisors of rat models undergoing controlled orthodontic forces, tooth movement was reduced and accelerated, respectively, whereas saline or no injection, and an absence of orthodontic forces served as controls. In these studies, histopathologic analysis and/or measurement of tooth movement by plaster casts revealed that tooth movement and the number of osteoclasts, Howship’s lacunae, and capillary vascularization was significantly increased in the NOS precursor groups and decreased in the NOS inhibitor groups. This led to the conclusion that NO plays a role in enhancing the rate of OTM in rat models (Akin et al., 2004; Hayashi et al., 2002; Shirazi et al., 2002).

In vitro studies on NO have shown that hydraulic pressure enhances NO production in cultured human PDL fibroblasts through nNOS (Nakago-Matsuo et al., 2000), whereas cyclic tension force activates NO production in human PDL cells through eNOS (Kikuiri et al., 2000). In vivo, the expression of eNOS and iNOS in the PDL decreases in an occlusal hypofunction model (Watarai et al., 2004). The presence of eNOS and iNOS have also been shown in dental pulpal tissues (Felaco et al., 2000; Lohinai et al., 1995), and increased levels of gingival iNOS during periodontal inflammation have been reported, as compared with noninfamed gingival tissue (Hirose et al., 2001; Lappin et al., 2000). Although iNOS is not ubiquitously present in tissues, immunohistochemical analyses have revealed that clinically healthy gingival tissue has detectable amounts of iNOS (D'Attillio et al., 2004).

The exact role of each NOS isoform currently remains unclear. Recently, however, all NOS isoforms were shown in a rat model to be involved in OTM with increased expression on the tension side, with nNOS being more involved in early OTM events. Niforoushan and Manolson (2009) performed immunohistochemical analysis on horizontal sections of the first maxillary molars of rats subjected to 3 and 24 hours of OTM. Their conclusion was that all NOS
isoforms are involved in OTM at some capacity, with different expression patterns between the tension and pressure sides. It is this type of study that raises interest regarding the role of NO at various time points in human teeth undergoing OTM, and brings to light the importance of extrapolating this research into human subjects.

Currently, the literature is much more abundant in studies involving the role of NO in OTM in animal models as opposed to human subjects. One human study demonstrated a role for gingival eNOS and iNOS during the early phases of OTM in human gingival tissue collected by means of gingivectomy and studied by immunohistochemical analysis (D'Attillio et al., 2004). In this study, levels of eNOS and iNOS expression were significantly greater in gingival tissue associated with teeth undergoing OTM than when compared to controls. It is argued, however, that gingival crevicular fluid (GCF), as opposed to gingival tissue, has greater diagnostic potential (Tözüm et al., 2007). The presence of NO has been detected in GCF in research pertaining to periodontitis and dental implant stability (Güncü et al., 2008); however, a large void still remains regarding the relationship between levels of NO in GCF in teeth undergoing OTM.

Genc et al. (2013) investigated the effects of low-level laser therapy (LLLT) on nitric oxide levels in the GCF of maxillary lateral incisors during retraction of these teeth. It was found that LLLT does not have a significant effect on NO levels during OTM. In this study, samples of GCF were taken at the maxillary lateral incisors while these teeth were already experiencing orthodontic forces; thus, a baseline was not established. It is therefore clear that the deficient area in the literature requiring attention is whether the levels of NO are altered in GCF in human teeth upon the application of an orthodontic force.
2.4 Gingival Crevicular Fluid

2.4.1 Introduction

Biomarkers involved in OTM can be observed using sampling from four different sampling procedures. These procedures include tissue samples (biopsy), serum, GCF, and saliva (Zainal Ariffin et al., 2011). It has been proposed by Zainal Ariffin et al. (2011) and others that GCF and saliva are the more superior sampling procedures due to their practicality, ease of sampling, and non-invasive nature of obtaining the samples.

GCF can be collected from the gingival sulcus surrounding the teeth. It exists as either a serum transudate or as an inflammatory exudate. The components within the fluid are derived from a variety of sources, and this fluid reflects the constituents of serum, contributions from the gingival crevice, and the cellular response in the periodontium (Lamster and Ahlo, 2007). More specifically, GCF contains constituents from the host such as molecules from blood, contributions from cells, and tissues of the periodontium that include the vasculature, epithelium, connective tissues, as well as immune and inflammatory cells that have infiltrated into the periodontal tissues. These host-derived constituents in GCF include specific markers of inflammation, tissue breakdown products, enzymes, cytokines, and interleukins (Lamster and Ahlo, 2007).

The traditional methods of collection of GCF include the use of small filter paper strips, micropipettes, and specialized appliances to isolate and collect the fluid from the gingival margin (Lamster and Ahlo, 2007). Clinically, the most appropriate method is the placement of methylcellulose filter paper strips into the sulcus. The benefit of this approach is its non-invasive nature, however it can be time consuming and technique sensitive. Care must be taken to ensure
there is no contamination from saliva, blood, or plaque, as it has been shown that plaque and saliva on a test strip can influence the volume of fluid that is collected on the strip (Griffiths et al., 1992). The amount of time that each site is sampled must also be standardized, and the typical sampling time is 30 seconds (Lamster and Ahlo, 2007).

2.4.2 GCF collection in OTM Studies

Historically, the remarkable diagnostic value of GCF was first acknowledged by Brill and Krasse (1958). They placed filter paper into the gingival sulcus of the teeth of experimental animals, and detected dye that had been injected systemically. Thereafter, in the 1970s, enzymes and other immune cells were detected (Attström and Egelberg, 1970; Goodson et al., 1974), and research utilizing GCF increased markedly when it began to be studied extensively in periodontal disease research (Lamster and Ahlo, 2007).

Aside from the years of research in the periodontal field utilizing samples of GCF, a variety of biomarkers involved in OTM have been detected in GCF samples. Alkaline phosphatase, which is a marker of active bone-forming cells, was shown to have higher levels in the tension region of teeth undergoing OTM than the compression side (Perinetti et al., 2002). Levels of RANKL and OPG in orthodontically-moved teeth have also been assessed. It was found that on the compression side, RANKL levels were up-regulated and OPG levels were down-regulated. It was also found that a decrease in tooth movement was associated with decreased RANKL and OPG level changes in older patients than compared to younger patients (Kawasaki et al., 2006; Nishijima et al., 2006).

It has been established in these and other studies utilizing GCF that composition and flow rate of GCF is not stable; it changes according to the state of the PDL. For example, during
OTM, the forces imparted on the PDL lead to cellular interactions that result in the synthesis of a variety of cellular messengers and inflammatory mediators. Cellular proliferation and differentiation are also initiated, and ultimately tissue remodeling results (Kavadia-Tsatala et al., 2002). These metabolic changes and subsequent alterations in the GCF composition and flow rate can then be analyzed through sampling of GCF during OTM, providing great insight into metabolic changes that are occurring.

Clinically, the proven usefulness of GCF sampling is complicated by the fact that orthodontic appliances create an area for plaque accumulation. Plaque increases the inflammatory mediators that are released thus having an effect on the acquired samples. Coincidently, the role of plaque must not be discounted when GCF samples are taken from the gingival sulcus of teeth engaged in orthodontic appliances.

2.5 Griess Reaction

The Griess reaction was originally described by Griess in 1879 and over the years many modifications to the original reaction have been described. The reaction measures one of the two stable breakdown products of nitric oxide, nitrite (NO$_2^-$), in biologic medium. The Griess Reagent System (Promega, 2009) is based on the reaction depicted in Figure 2, which combines two reagents: 1) Sulfanilamide and 2) N-1-napthylethlyenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. The combination of these two reagents creates an azo compound which turns a visible purple colour, and the absorbance can be read at the peak absorbance of this azo compound, 540 nm (Green et al., 1982; Promega, 2009).
**Figure 2:** Chemical reactions involved in the Griess Reagent System (Promega Corp). Reprinted with permission from (Promega, 2009).

**CHAPTER 3: MATERIALS AND METHODS**

**3.1 Inclusion Criteria**

Ethical approval was obtained from the University of Toronto Health Sciences Research Ethics Board (#26812). Participants were recruited from the Graduate Orthodontics Clinic at the University of Toronto, and were selected based on the following inclusion criteria:

1. Males between the ages of 10 and 20 years, treatment planned to be undergoing orthodontic therapy with fixed-edgewise appliances. Males were selected on the basis of “decreased” hormonal changes throughout the course of the study as compared to females of pubertal age (Duckles and Miller, 2010).

2. Dental malocclusion indicating orthodontic treatment, with mild/moderate crowding as defined by Carey’s Analysis exhibiting no greater than 6 mm of space discrepancy per arch.

3. Treatment planned for non-extraction treatment.
4. Healthy systemic condition.

5. Non-smoker, no alcohol consumption.

6. No use of anti-inflammatory drugs in the month preceding the beginning of the study (D'Attillio et al., 2004).

7. Absence of periodontal disease as defined by probing depth values no greater than 3mm, no loss of attachment >2mm (measured as the distance from the bottom of the sulcus to the cemento-enamel junction), and an absence of periodontal bone loss as revealed from bitewing radiographs (D'Attillio et al., 2004).

8. Oral hygiene must be fair/good as determined by a Modified Gingival Index (MGI) (Lobene et al., 1986) and Plaque Index (PI) (Silness and Loe, 1964). Adequate oral hygiene must be maintained by participants throughout the course of the study, and MGI and PI were recorded for each sampled site at each sampling time point. MGI and PI are described in Appendix A. If at any time a patient exceeded scores of MGI = 2 or PI = 2 they would be excluded from participation in the study. This did not occur, however.

Informed consent was obtained verbally and in writing from all included subjects or their parents if the subjects were below the age of 18. The patient information and consent form that was read and signed by all participants is included in Appendix B. Participants were compensated twenty dollars for returning to the orthodontic clinic for one additional visit.
3.2 Sampling Procedure

3.2.1 Sites Sampled

Samples of GCF were taken immediately prior to bonding with fixed-edgewise appliances (braces), 1 hour after bonding and insertion of a light (0.014”) NiTi aligning maxillary archwire, and 3-4 days following bonding.

Upon orthodontic appliance placement in a crowded arch, teeth tend to procline in order to align themselves (Fleming et al., 2012; Weinberg and Sadowsky, 1996). In general, therefore, the buccal surfaces of teeth (especially anterior teeth) act as the “pressure” side whereas the lingual surfaces act as the “tension” side. To investigate potential difference in NO levels between the pressure and tension sides, GCF samples were collected on both the buccal and lingual sides of the maxillary central incisors, and first and second molars provided these teeth were not severely displaced outside of the archform (>3mm) and were planned to be included in the initial archwire placement. The maxillary second molars were selected to serve as controls and therefore the archwire did not engage them. The second molar, either the left or the right, was bonded with a molar tube, while the other side remained non-bonded to serve as a control for plaque accumulation due to a bonded attachment. In all twelve sites (six buccal and six lingual) were sampled at each time point for each patient (Figure 3).
Figure 3: Sites of GCF collection. Arrows point to the buccal and lingual sampling sites of the central incisors, first molars, and second molars. The second molars served as bonded (with an attachment), and non-bonded (without an attachment) controls.

Participants were advised to take Acetaminophen (but not NSAIDS) as needed for pain relief during the course of the study, due to the documented effects that anti-inflammatory drugs have on OTM as well as on NO levels (Arias and Marquez-Orozco, 2006; Qandil, 2012). Participants were also asked not to consume foods known to be high in nitrates, such as processed meats, the day before sampling was to take place.

An increase in nitrite levels as early as 1 hour after elastic separator placement was observed in a pilot study with elastic separators placed on the mesial and distal surfaces of maxillary first molars of 3 male patients (Appendix C). This finding was similar to a previously published study utilizing a rat model (Yoo et al., 2004). It has also been clearly documented that bone remodeling of the socket is present after 2 days of sustained orthodontic forces and continues beyond the 3rd and 4th day of force application, as reviewed in Proffit et al. (2007). NO has been shown to play a role in both osteoblastic bone formation as well as osteoclastic bone
resorption, processes involved in bone remodeling (van't Hof and Ralston, 2001). Therefore, for this current study, samples were collected at three time points: an initial baseline (T0), 1 hour after archwire placement (T1), and 3-4 days after a sustained orthodontic force (T2).

3.2.2 GCF Sampling Procedure

The sampling procedure for NO in GCF was adapted from Güncü et al. (2008). The sampling site was prepared by gently removing supragingival plaque followed by drying and isolation with sterile gauze or cotton rolls. Standardized paper strips (Periopaper, Oraflow) were calibrated on an electronic volume quantification device (Periotron 8000) which is used for determination of the obtained GCF volume. (It should be noted that calibration of this device was performed prior to sampling by quintuplicate readings and verified at regular intervals. Please refer to Section 3.3 ‘Periotron Unit Calibration’ for a description). The Periopaper strips were inserted into the gingival sulci to a standardized depth of 1 mm at each site, for a standardized time of 60s. The sampling protocol was consistent among all sites regardless of probing depth, and any samples with blood, plaque or saliva contamination were discarded. Periopaper strips were then measured in Periotron units after sample collection, and converted to microliters using a previously constructed Periotron unit volume calibration curve (Section 3.3.2). Paper strips were immediately placed into sterile Eppendorf tubes and immersed in Liquid Nitrogen to prevent evaporation. Once all twelve samples were collected for each sampling time point and placed in Liquid Nitrogen, the samples were then transported to a freezer to be stored at -30°C until the day of laboratory analysis.
3.3 Periotron Unit Calibration

3.3.1 Periotron 8000 Unit

The Periotron 8000 (Pro-Flow Inc., Amityville, NY, USA), depicted in Figure 4, quantifies the volume of GCF collected on filter paper. It measures the capacitance of a wet filter paper strip that is inserted in between the jaws of the instrument (Ciantar and Caruana, 1998). Essentially, opposing charges on the jaws of the instrument create electric fields that induce polarity of the molecules. The higher the number of polar molecules between the jaws of the Periotron, the larger the capacitance, and thus a larger Periotron score will be displayed. When the unknown fluid is placed on a filter paper strip and a Periotron score is displayed, this unknown volume can then be determined from calibration graphs that are constructed. These calibration graphs use accurately measured known quantities of fluid that are used to reveal the volume that the Periotron score represents (Ciantar and Caruana, 1998).

**Figure 4:** Periotron 8000 Unit (Pro-Flow Inc., Amityville, NY, USA) used to quantify the amount of volume collected in each GCF sample. Arrows point to 1) The site where PerioPaper strips containing GCF samples are inserted, and 2) The display screen where the volume collected is revealed in Periotron units.
3.3.2 Calibration of the Periotron 8000 Unit

The Periotron 8000 Unit was calibrated once per month during the time period that GCF samples were actively being collected. Calibration was completed as per previous recommendations (Chapple et al., 1999; Ciantar and Caruana, 1998).

Calibration of the Periotron 8000 unit took place in the Graduate Orthodontics clinic, Faculty of Dentistry, University of Toronto, where the GCF sampling took place. Before every measurement, the Periotron Unit was dried and re-set to zero using a dry PerioPaper (OraFlow, Inc.) strip. Double distilled water was applied to each PerioPaper strip, using a calibrated pipette applied to the edge of the strip while being held at a 45º angle. Within 10 seconds after the fluid application to the filter paper, the PerioPaper strip was inserted in between the jaws of the Periotron unit 0.5-1 mm from the orange half of the paper. The volumes that were tested ranged from 0.1 to 1.1 μl, and were applied in 0.1 μl intervals. For each volume tested, this procedure was repeated 5 times and the average Periotron score was recorded and displayed graphically. The average Periotron scores over the multiple times the Periotron unit was calibrated are depicted in Figure 5. The calibration curve that corresponded with the closest time point that samples were collected was utilized when the calculations of total nitrite levels was completed.
3.4 Laboratory Analysis

The laboratory analysis protocol outlined in sections 3.4.1 and 3.4.2 was adapted from Promega (2009).

3.4.1 Nitrite Standard Reference Curve

A Nitrite Standard reference curve was prepared for each assay that was completed in order to achieve accurate quantification of NO$_2^-$ levels.

1. A 50 µM nitrite solution was prepared by diluting the 0.1M Nitrite Standard solution.
2. 3 columns of a sterile 96-well plate were designated for the Nitrite Standard Reference Curve.
3. 6 serial twofold dilutions were performed, with the volume in each well set at 50 µL, and Phosphate buffered saline (PBS) being utilized as the matrix. The final concentration in each well in rows A-H was 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0µM, where no

Figure 5: Average Periotron Unit Scores for known volumes of ddH$_2$O. The average was taken over four calibrations, completed once per month over the period that samples were collected and the Periotron 8000 Unit was in use. Each volume was repeated five times and the average was recorded.
Nitrite Standard was added to the final well, and the 50 μM Nitrite Standard solution was plated in row A. A schematic is shown in Figure 6.

4. A Nitrite Standard reference curve was created for each assay completed, and was utilized in the calculation of total nitrite levels.

<table>
<thead>
<tr>
<th>Nitrite Standards NO₂⁻ Conc (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6 7 8 9 10 11 12</td>
</tr>
<tr>
<td>A 50 50 50</td>
</tr>
<tr>
<td>B 25 25 25</td>
</tr>
<tr>
<td>C 12.5 12.5 12.5</td>
</tr>
<tr>
<td>D 6.25 6.25 6.25 (Plated samples)</td>
</tr>
<tr>
<td>E 3.13 3.13 3.13</td>
</tr>
<tr>
<td>F 1.56 1.56 1.56</td>
</tr>
<tr>
<td>G 0.78 0.78 0.78</td>
</tr>
<tr>
<td>H 0 0 0</td>
</tr>
</tbody>
</table>

**Figure 6:** Schematic of Nitrite Standards and experimental samples plated in a sterile 96-well plate. Columns 1-3 and rows A-H were utilized for nitrite standards plated in triplicate, and the remainder of the wells were utilized for experimental samples.

### 3.4.2 Measurement of Nitrite in Experimental Samples

Seventy μL of PBS was added to all GCF samples (eppendorf tubes containing PerioPaper strips). All samples were mixed vigorously for 20 seconds each, and allowed further equilibration to room temperature. The two reagents involved in the Griess reaction, Sulfanilamide and NED solution were also allowed to equilibrate to room temperature, 15-30 min, after removal from storage at 4°C. Fifty μL of all GCF samples were plated in a 96-well plate along with 50 μL of sulfanilamide solution then being added to all experimental samples and nitrite standards, and the plate was incubated for 5-10 minutes, protected from the light. Fifty μL of NED solution was then added to all wells, and again protected from light it was incubated.
5-10 minutes. The absorbance was then read by a plate reader within 30 minutes, at 540 nm which is the peak of the absorbance spectrum of the coloured azo compound formed by the Griess reaction. Utilizing the absorbance values, the total nitrite levels in the GCF samples were then calculated.

3.5 Pilot Study

An initial pilot study was undertaken to: a) Ensure that the protocol is sound and an adequate volume of collected GCF is measurable, b) Ensure that NO is detectable in the collected GCF and saliva, and, c) Serve as a basis for designing the protocol as to what time points the samples will be taken on the subjects. Elastic separators were placed mesial and distal to the first molars of three male volunteers (Graduate Orthodontic residents and/or faculty). GCF samples were collected with Periopaper strips (Oraflow Inc.) at a variety of time points: Immediately prior to the placement of separators, at 1h post-separator placement, and 3 days (two subjects) or 1 week (one subject) following the placement of the separators. The levels of NO in the GCF and saliva samples were then measured. The amount of measurable GCF at each individual time point of OTM was analyzed to determine primarily whether there is a measurable change in the quantitative levels. The results of this pilot study are outlined in Appendix C, and served as a basis for appropriate study design for the main study.
Thirteen male adolescents (mean age 14.0 years; range 11-18 years) participated in the study. Samples were collected at three time points (T0, T1 and T2). As there was no significant difference between the samples collected after 3 or 4 days, these were grouped together as the third sampling time point, T2.

In order to analyze the data, teeth sampled were divided into three groups: 1) Anterior pressure teeth (the two central incisors), 2) Posterior pressure teeth (the first molars), and 3) Control teeth (the second molars). A significant difference in nitrite concentration was not observed within each of these tooth groups (i.e., between the two central incisors within each patient, between the first molars, and between the non-bonded and bonded control teeth), allowing the formation of these groups for statistical analysis. The values for the buccal and lingual surfaces of each of these groups were analyzed separately.

Statistical analysis was conducted implementing comparative t-tests between tooth groups at each time point, and between time points within each tooth group, for both the buccal and lingual surfaces. Additionally, Generalized Linear Mixed Models (or GLMMs) were utilized to analyze the data, with NO concentration in GCF as the outcome variable, and treatment, time, and age as the independent variables. GLMM is an extension of linear mixed models to allow response variables from different distributions. Median values of the total NO concentrations in GCF of the buccal surfaces showed significantly higher NO concentrations at the buccal surfaces of the anterior pressure teeth at T1, when compared with the posterior pressure teeth and the controls at T1 (p=0.025) (Tables 2 and 3, Figure 7). A comparison between baseline values obtained at T0 and the general decrease in NO concentrations at T1 for the buccal surfaces and
general increase measured at T2 for all groups was not statistically significant (Table 2). Comparative $t$-tests between the posterior pressure teeth and the controls did not demonstrate a significant difference at any time points ($p>0.05$) (Table 3). In summary, the buccal surfaces of the anterior pressure teeth showed significantly higher nitrite levels when compared to the posterior pressure and control teeth at T1.

**Table 2: Total Nitrite Concentration for Buccal and Lingual Surfaces**

(C=Control tooth group, PP=Posterior pressure tooth group, AP=Anterior pressure tooth group)

<table>
<thead>
<tr>
<th></th>
<th>Buccal Surfaces Median Nitrite Concentration (μM) (Q1, Q3)</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
<td>T1</td>
</tr>
<tr>
<td>C</td>
<td>134.7 (49.3,282.4)</td>
<td>67.3 (42.5,147.0)</td>
</tr>
<tr>
<td>PP</td>
<td>106.9 (43.2,201.8)</td>
<td>57.6 (28.2,147.6)</td>
</tr>
<tr>
<td>AP</td>
<td>155.3 (13.6,347.6)</td>
<td>124.4 (56.8,263.8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Lingual Surfaces Median Nitrite Concentration (μM) (Q1, Q3)</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
<td>T1</td>
</tr>
<tr>
<td>C</td>
<td>166.0 (105.6,281.9)</td>
<td>175.3 (100.4,254.2)</td>
</tr>
<tr>
<td>PP</td>
<td>181.0 (75.3,379.0)</td>
<td>125.1 (58.1,237.7)</td>
</tr>
<tr>
<td>AP</td>
<td>216.3 (71.6,524.3)</td>
<td>240.6 (40.1,379.3)</td>
</tr>
</tbody>
</table>

**Table 3: P-values from Comparative t-tests between Tooth Groups**

(C=Control tooth group, PP=Posterior pressure tooth group, AP=Anterior pressure tooth group, * = statistical significance $p<0.05$)

<table>
<thead>
<tr>
<th></th>
<th>BUCCAL</th>
<th>LINGUAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C vs. AP</td>
<td>C vs. PP</td>
</tr>
<tr>
<td>Main Effect</td>
<td>0.0251*</td>
<td>0.7414</td>
</tr>
</tbody>
</table>
Figure 7: Median total nitrite concentrations in GCF at each time point (T0, T1, T2) for the buccal surfaces of the tooth groups indicated. The middle band within the box represents the median, the upper and lower portions of the box represent the interquartile range, the extended bars represent 1.5 times the interquartile range, and circles represent outliers. AP=Anterior pressure teeth, PP=Posterior pressure teeth, and C=controls.

In contrast to the NO levels observed at the buccal sites, the lingual surfaces of sampled sites did not demonstrate statistically significant differences between tooth groups and also between time-points within each group (Tables 2 and 3, Figure 8). Additionally, for both the buccal and lingual sites, the posterior pressure teeth (first molars) did not demonstrate significant differences when compared with the second molar controls (Table 3).
Figure 8: Median total nitrite concentrations in GCF at each time point (T0, T1, T2) for the lingual surfaces of the tooth groups indicated. The middle band within the box represents the median, the upper and lower portions of the box represent the interquartile range, the extended bars represent 1.5 times the interquartile range, and circles represent outliers. AP=Anterior pressure teeth, PP=Posterior pressure teeth, and C=controls.

An additional interesting finding was found when the results were controlled for age. The values for the participants were arbitrarily divided into two age groups, an older age group (14-18 years) and a younger age group (11-13 years), to investigate if age played a role in the findings. In general, there were no significant findings between the age groups. However, at T1, the older age group demonstrated higher anterior pressure nitrite levels when compared to the younger group for the lingual surfaces (p<0.05). The values for the other surfaces demonstrated a slightly similar trend however it did not reach statistical significance. Table 4 outlines these comparative statistics, showing this significant p-value in the “age” row. The “main effect” refers to the overall difference noted for the buccal surface sites as mentioned. When the results were controlled for time it was shown that there was no statistically significant difference between the values obtained from sampling after either 3 or 4 days.
### Table 4: Comparative statistics (P-values) with reference to time points, treatment, and age

(* is for statistical significance P<0.05)

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<tr>
<th></th>
<th>BUCCAL</th>
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<tr>
<td></td>
<td>17/27 vs. 11/21</td>
<td>16/26 vs. 11/21</td>
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<tr>
<td>Main Effect</td>
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<tr>
<td>Age</td>
<td>0.9143</td>
<td>0.0499*</td>
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### Chapter 5. Discussion and Conclusions

In this study, the levels of nitrite, a stable and non-volatile breakdown product of NO, were measured in the GCF of specific maxillary teeth of male patients before and after the application of light continuous orthodontic forces. GCF samples were successfully collected at an adequate volume with measurable levels of nitrite and differences in the nitrite levels at specific sites of the anterior versus posterior teeth were shown.

The study of biomarkers of OTM is widespread and involves *in vitro* and *in vivo* studies. Although there are numerous animal studies on the role of NO in OTM, few studies have been performed on humans. The sampling methods that have been used by other investigators to examine the levels of NO in humans include the collection of gingival tissue by means of a gingivectomy and immunohistochemical analysis, and analysis of salivary and GCF levels of NO (D’Attillio et al., 2004; Güncü et al., 2008; Parwani et al., 2012). Collection of GCF samples was selected because of the many advantages that this sampling method provides. GCF composition is quite dynamic and changes according to the state of the PDL. Metabolic changes e.g., cellular proliferation and differentiation of cells in the PDL during OTM (Kavadia-Tsatala et al., 2002), are reflected in the subsequent alterations in the GCF composition and flow rate. Biomarkers of OTM such as alkaline phosphatase, RANKL, and OPG have been successfully studied utilizing
GCF samples, with enough sensitivity to differentiate between the pressure and tension sides during OTM (Kawasaki et al., 2006; Nishijima et al., 2006; Perinetti et al., 2002). GCF sampling is also an easy and non-invasive sampling procedure. One major challenge with GCF sampling, however, is that the collected volume is extremely small. A number of measures were adopted to maximize the amount of GCF volume collected. Samples were “snap frozen” in liquid nitrogen immediately after collection to prevent evaporation and the Periopaper strips were left in place for 60s instead of 30s which was implemented in another study (Güncü et al., 2008). Precautions were also taken to ensure that participants maintained a healthy periodontal condition free from inflammation as it is known that NOS is activated in neutrophils in response to inflammatory stimuli such as plaque (Moilanen and Vapaatalo, 1995). Since final samples were collected only a few days after bracket placement and there was no statistical difference between the bonded and non-bonded second molar controls, changes in NO due to inflammation can be successfully ruled out in this study. The data indicate that measurable levels of NO were successfully and consistently obtained in the gingiva of teeth following orthodontic force application.

The GCF volumes collected at each site were in a similar range to the those obtained in other GCF collection studies, where the volume collected was in the range of 0.1-0.8 µL compared to the 0.1-1.2 µL range found in the current study (with a few elevated outliers) (Güncü et al., 2008; Perinetti et al., 2013). The nitrite concentration obtained at each site in this study was also in a similar range to the peri-implant sulcus fluid (PISF) samples collected on healthy patients in the aforementioned study (Güncü et al., 2008). A previous study investigating the role of low-level laser therapy (LLLT) on OTM in healthy patients utilized similar sampling methodologies as the ones used in the current study showed concentrations of NO that were slightly lower than those found in the current study and in Guncu et al. (2008) (Genc et al.,
Overall, the GCF sampling methodology was successful in collecting an adequate volume of GCF, with levels similar to studies published of this kind.

The selection of time points for gingival sampling in the current study was based on documented evidence from both animal and human studies of cellular and tissue reactions subsequent to the application of an orthodontic force. Additional sampling time points in between one hour and 3-4 days would have been useful to gain a better understanding of the exact early role that NO plays in OTM, however limitations in clinic closure times and patient attendance presented difficulties. Patient and parent cooperation was essential in this study, especially in terms of oral hygiene requirements, diet and avoidance of NSAIDS, as well as attendance. Participants were compensated twenty dollars for their time and transportation for the one additional visit to the Faculty of Dentistry, Graduate Orthodontics clinic. Any additional visits to the faculty would pose challenges when recruiting patients to participate in the study, as some of the patients travel a great distance to attend the orthodontic clinic and often had to be accompanied by a parent. The difficulty in patient recruitment was a major determining factor in the smaller sample size and the limited sampling time points.

Differentiation between the pressure and tension sides was made based on the generally accepted paradigm that upon orthodontic appliance placement in a crowded arch, teeth tend to procline in order to align themselves (Fleming et al., 2012; Weinberg and Sadowsky, 1996). In general, therefore, the buccal surfaces of teeth (especially anterior teeth) act as the “pressure” side whereas the lingual surfaces act as the “tension” side. In this study, through standardization of malocclusions, 12 of the 13 participants (92.3%) presented with a mildly crowded dentition, thus the incisors were indeed undergoing proclination during initial alignment. For the one participant who presented with mild spacing, the anterior teeth would most likely not move in a
buccal/lingual direction, and retroclination of these teeth in this spacing case, which would reverse the pressure/tension sites, is highly unlikely. Therefore, for the most part, the initial alignment of teeth for patients in this study involved movement of anterior teeth in the labial direction, thus defining the pressure side as being on the buccal, and the tension side as being on the lingual.

NO has been shown to have biphasic effects with regard to the role of NO in osteoclastic bone resorption. Low concentrations of NO have been shown to potentiate IL-1 induced bone resorption (Ralston et al., 1995) whereas high concentrations of NO have been shown to inhibit osteoclastic formation and activity (van't Hof and Ralston, 2001). Constitutive production of NO within osteoclasts has been suggested to be essential for osteoclast function, based on experimentation where NOS inhibitors were shown to inhibit the activity and motility of isolated osteoclasts (Brandi et al., 1995). In the current study, although it was difficult to accurately extrapolate these animal findings to those in humans, NO levels that could constitute “higher” and “lower” concentrations of NO were obtained. The buccal surfaces of the anterior pressure teeth demonstrated elevated nitrite levels in the range of 124.4 μM, low in comparison to the higher delayed elevation at the lingual surfaces 3-4 days later of 346.1 μM. These “low” and “high” concentration values suggest that the findings in this study correlate with the findings in animal models, where lower concentrations of nitrite are present on the pressure side potentiating bone resorption, and higher concentrations are present on the tension side, inhibiting osteoclastic activity and formation. Certainly, further investigation into what truly constitutes low and high concentrations in humans is indicated.

At the cellular and molecular levels, the higher NO concentration observed at the buccal sites of the anterior pressure teeth at T1 when compared to posterior pressure teeth and controls
can be attributed to the role of NO in increasing microvascular permeability (Rumbaut and Huxley, 2002) that allows for the influx of monocytes into blood vessels, which are processes critical for bone remodeling (Alberts et al., 2002). Ultimately, bone remodeling results in the movement of the tooth within the bony socket, thus NO appears to play a role in the early initiation of OTM. These results were also shown in rat models as the early changes that occur within the vascular periodontal ligament at one hour after orthodontic force initiation (Yoo et al., 2004).

The initial NO concentrations measured at baseline (T0) for the buccal surfaces were found to be slightly higher than the values obtained at T1, although this observation did not reach statistical significance (Table 2). A possible explanation for this finding is that the orthodontic bonding procedures, which involve rinsing and significant drying, played a role in decreasing these NO concentrations at T1. The bonding procedures are only undertaken on the buccal surfaces, thus this effect was not observed on the lingual surfaces.

A possible reason for the lack of a significant difference in NO levels between the posterior pressure and control teeth in this study could be that very low orthodontic forces were imparted on molars with the light NiTi initial aligning archwire. An increase in NOS activity, which is directly proportional to NO concentration, has been documented on the pressure side of maxillary molars in rats one hour after force application (Yoo et al., 2004), in contrast to the current study where the molars did not show a significant change. In rat models, however, in contrast to humans, the incisors typically act as the anchors and the molars are the teeth that are moved (Yoo et al., 2004). In OTM studies using rat models, molars are typically mesialized or “pulled” with closed coil springs. These coils also generate a much higher force compared to those created by the light NiTi wires used in the current study (Sarul et al., 2013). Use of a coil
spring is not practical to initiate orthodontic tooth movement in humans due to this reason. Future investigations into whether NO levels vary depending on the magnitude of the force applied would be useful. One possible approach to applying an increased orthodontic force to the maxillary molars is to study NO levels before and after the use of a headgear appliance, which delivers a heavier and measurable orthodontic force.

Another interesting finding was that when the results were arbitrarily divided into two age groups, an older age group (ages 14-18) and a younger age group (11-13) to determine any possible effects of age. In general there was no significant difference among the time points and sites samples with regard to the older and younger age groups. However, at T1, the older age group demonstrated higher anterior pressure nitrite levels when compared to the younger group for the lingual surfaces only (p<0.05). This result is likely attributed to the elevated levels of growth hormone that are present in the older age 14-18 age group (Rogol et al., 2002), as it has been shown that growth hormone increases NO production (Duckles and Miller, 2010). It is not clear as to why this finding was only at that specific site as opposed to generalized elevated levels for the entire older age group. This finding does not impact the results of the overall study, since comparisons were made within each patient from obtained values of the pressure teeth when compared to the control teeth. The finding was interesting, nonetheless.

In summary, the results showed that the pressure side of central incisors demonstrated significantly elevated nitrite levels one hour after placement of an orthodontic appliance, when compared to posterior pressure teeth and control teeth. The standardization of malocclusions in this study did allow for the identification of defined pressure and tension sites, without the possibility of exact force calculation due to the creation of an indeterminate force system (Proffit et al., 2007). The GCF sampling strategy and methods employed in this study successfully
demonstrated that NO can be consistently detected in the GCF of healthy subjects, and that NO
appears to play a role in the early stages of OTM. This knowledge has developed an improved
understanding of NO expression following application of light orthodontic tooth moving forces
in humans. These methods and results will be useful for further investigations that could explore
NO activity at a molecular level in humans. This knowledge is useful in developing strategies to
improve the efficiency of OTM.
This study raises several questions that warrant further investigation.

i. **Is a force gradient present and necessary to effect changes in NO levels?** It was hypothesized that the reason a significant difference between nitrite levels at the posterior pressure teeth and the controls was not found was due to the very light continuous forces present. Previous studies utilizing nickel titanium coil springs in animal models found elevated NOS activity at the molars (Yoo et al., 2004). The application of greater forces, such as the use of a maxillary headgear appliance, could potentially lead to the understanding of whether NO levels vary depending on the amount of force applied.

ii. **Are the findings in rats consistent with humans at a cellular and molecular level?** NO has been shown to decrease the RANKL/OPG equilibrium leading to positive bone formation due to a decreased recruitment of osteoclasts. It was hypothesized that the delayed increase in nitrite noted on the tension side of the anterior pressure teeth accounts for this finding, but confirmation utilizing human tissue where possible would be ideal.

iii. **Would additional sampling time-points have provided further insight into the early role of NO in OTM?** Collecting additional samples between 1 hour and 3-4 days following bonding may have provided additional insight into how NO levels change over the first few days following the application of light orthodontic forces.

iv. **Were the “low” and “high” concentrations of NO found in this study comparable to the observations made in rat models?** Low concentrations of NO have been shown to potentiate bone resorption, whereas “high” concentrations have been shown to inhibit osteoclastic bone resorption (van't Hof and Ralston, 2001). The results found in this study
seem to correlate with these findings, but further investigation into what truly constitutes “low” and “high” concentrations in humans is warranted.


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APPENDIX A – MODIFIED GINGIVAL INDEX AND PLAQUE INDEX

The Modified Gingival Index and Plaque Index recorded for each patient at each sampling time point are listed below. Maximum acceptable scores for patients were ≤ 2 (MGI) and ≤ 2 (PI), where patients would be excluded from participation in the study if they exceeded these scores over the course of sampling.

A.1 Modified Gingival Index

Adaptation of Modified Gingival Index (MGI) from Lobene et al. (1986).

A score was assigned for each site sampled based on the following criteria:

0 Absence of inflammation

1 Mild inflammation; slight change in color, little change in texture of any portion of but not the entire marginal or papillary gingival unit

2 Mild inflammation; criteria as above but involving the entire marginal or papillary gingival unit

3 Moderate inflammation; glazing, redness, edema, and/or hypertrophy of the marginal or papillary gingival unit

4 Severe inflammation; marked redness, edema and/or hypertrophy of the marginal or papillary gingival unit, spontaneous bleeding, congestion, or ulceration

A.2 Plaque Index

Plaque Index scores were adapted from Silness and Loe (1969).

A score was assigned for each site sampled based on the following criteria:

0 No plaque
1. A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface.

2. Moderate accumulation of soft deposits within the gingival pocket, or on the tooth and gingival margin which can be seen with the naked eye.

3. Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

**APPENDIX B – PATIENT INFORMATION AND CONSENT FORM**

All patients and their parents as applicable gave consent verbally and in writing. The patient information and consent form is found on the following pages.
Project Title: Nitric Oxide Levels in Gingival Crevicular Fluid during Orthodontic Tooth Movement
Principle Researcher: Dr. Heather Ford

Why are you being given this form?
You are being asked to participate in a research study while you are currently undergoing orthodontic treatment.
The information in this form is intended to help you understand exactly what we are asking of you, so that you can decide whether or not you wish to participate in this study. Please read this consent form carefully and ask all the necessary questions you may have before deciding whether or not to participate in this study. Your participation is entirely voluntary and a decision not to participate will not in any way affect any future treatment.

Why is this study being done?
The study will investigate the changes in levels of Nitric Oxide in gingival crevicular fluid (GCF) (or, the fluid found in your “gums”) before, and during the application of an orthodontic force. Nitric oxide is an important cell signaling molecule found in humans, and it can be detected in the fluid found within human gingiva, or gums. Nitric oxide has also been detected in saliva, and for that reason a saliva sample will also be taken before and after you are bonded with braces.

Nitric oxide has been shown in animal studies to increase the rate of orthodontic tooth movement when its levels are increased, and decrease the rate of orthodontic tooth movement when its levels are decreased. It has yet to be determined, however, whether the levels of nitric oxide in GCF or saliva increase or decrease during orthodontic tooth movement in humans. This study wishes to quantify the levels of nitric oxide with the application of an orthodontic force on humans.

This type of study could potentially lead to future studies on increasing the rate of orthodontic tooth movement for patients. Traditional orthodontic treatment with fixed appliances, or braces, typically takes 12 to 36 months, and studies that potentially may decrease this treatment time would greatly benefit patients, their parents, and orthodontists. This study intends to include the participation of approximately 20 patients in the University of Toronto, Faculty of Dentistry, Graduate Orthodontics Clinic.

What will you be asked to do?
During your regularly scheduled orthodontic visits (except for one), the principle researcher, Dr. Heather Ford, will take samples of gingival crevicular fluid, or the fluid found within your gums, as well as saliva, at specified time points. The sampling method involves placing a small point of paper 1 mm into the space in between your teeth and your gums, and allowing the fluid to absorb into the paper for 30 seconds. These samples will be taken at two sites in your two front teeth, or central incisors, and at two sites each in your first and second molars. The sampling process is entirely painless and non-invasive, and will not cause harm to your gums. Additionally, we will have you expectorate or “spit” saliva into a sterile container to collect the saliva samples. Each sequence of sampling should take around 30 minutes in total.
When will samples be taken?
Samples will be taken right before (on the same day) you have your upper braces placed for the first time. Then, the same sequence of samples will be taken one hour after your braces are placed, and then three days following the placement of braces. The visit that occurs at three days after the braces are placed will likely not be a scheduled appointment with your assigned student orthodontist, however this appointment will serve as a great opportunity for you to ask any questions you may have about your braces and for Dr. Ford to go over oral hygiene procedures again with you. Additionally, you will be compensated for your attendance at this visit.

Where will the study take place?
The study will take place in the Graduate Orthodontics Clinic, University of Toronto during your regularly scheduled orthodontic appointments. You will be asked to visit the clinic for one additional visit, for which you will be compensated.

Will I be compensated for my participation in this study?
Participants will be compensated $20 for their participation in this study to cover the cost of transportation and/or inconvenience. This compensation will be given to participants upon completion of the study (when you return for the second appointment). There are no additional costs incurred to the subject for participation in this study.

Will my participation in this study affect my regular orthodontic treatment?
Your participation in this study will not affect your regular orthodontic treatment in any way. Your student orthodontist will still be using the same type of braces and wires as they have treatment planned for your individual case. They will also see you for your regularly scheduled appointments, and your treatment will not be altered in any way.

Who is eligible to participate in this study?
We are seeking male participants undergoing orthodontic treatment (braces) at the University of Toronto, Faculty of Dentistry, Graduate Orthodontics clinic.
The criteria used for selection includes participants that:
- Have been treatment planned by their assigned student for non-extraction treatment with braces
- Have mild/moderate crowding
- Have a healthy systemic condition
- Are non-smokers and do not consume alcohol
- Have not used anti-inflammatory drugs, such as Ibuprofen (or “Advil”) in the month prior to the study and during the study (You will be recommended to take Acetaminophen (or “Tylenol”) for pain relief if needed after the braces are placed)
- Do not have gum disease, or “periodontal disease”
- Have good oral hygiene
What are the potential harms, risks, and benefits?

There are no potential harms or risks associated with this study. These types of samples are routinely taken in periodontal research, or research associated with gum disease. The sampling is entirely painless and non-invasive, and does not alter or harm your gums in any way.

The potential benefits of your participation in this study are great. Not only will you be contributing to the body of knowledge regarding orthodontic tooth movement, as well as potentially leading to studies that may decrease treatment time for braces, but you will be compensated $20 for your participation. You will also benefit from constant reinforcement of the importance of proper toothbrushing and flossing techniques, thus decreasing your risk for cavities or gum disease.

What are my alternatives to participating in the project?
You may choose not to participate in the study and still receive regular orthodontic treatment. You may also withdraw from the study at any time.

Can I have access to the results of the study?
The M.Sc. Thesis that will result from this research will be made publicly available. As well, should the results of the study be published, you may certainly have access to the publication. Please let the principle researcher, Dr. Heather Ford, know that you would be interested in owning a copy of the publication.

Privacy and Confidentiality

Dental and medical records that contain your identity will be treated as confidential in accordance with the Canadian Personal Information Protection and Electronic Document Act and provincial privacy laws. Your identity will be kept confidential at all times, except where disclosure is required by law.

As part of this research, the study doctor will collect the results of your study-related procedures and may also access your personal medical records for health information such as past medical history and test results. Information collected from the study site will not contain your name. Your dental/orthodontic records, which include your name, may be inspected at the study site by the monitor(s), auditor(s), representatives of the research ethics committees (an independent committee that reviewed the ethical aspects of this study to help protect the rights and welfare of study participants). This inspection is to verify the accuracy of study records and of the clinical trial procedures and/or data without violating your confidentiality and to the extent permitted by the applicable laws and regulations. This information may also be reported to Health Canada in the case of adverse events observed in the course of the research. If the results of the study are published, your identity will remain confidential. Data resulting from this study shall be retained at University of Toronto in controlled electronic and hardcopy records. You have the right to check your study records and request changes if the information is not correct.

While every effort will be made to protect the privacy of your information, absolute confidentiality cannot be guaranteed. However, this does not limit the duty of the researchers and others to protect your privacy. By signing this Patient Information and Consent Form, you consent to the collection, access, use and disclosure of your information as described above.
You have the right to change your mind
Your participation is entirely voluntary. You can refuse to take part in this project at this point or withdraw from it at any time during the study, without incurring any penalty or loss of benefits to which you are otherwise entitled. If you decide to withdraw during the course of the study, any clinical information collected from you until then may still be used for the purpose of the study. The study orthodontist/dentist may also withdraw you from the study if you do not follow the instructions you received from the study personnel, if the study orthodontist/dentist feels it is in your best interests to be withdrawn, if the study sponsor discontinues the study, or for administrative reasons. You may be withdrawn without your consent, but the study orthodontist/dentist will explain to you why.

Who to contact if you have any further concerns or questions?
Any further questions or concerns may be directed to Dr. Heather Ford (416) 979-4912 x 2. Dr. Ford can also be reached at dr.hford@gmail.com.

Ethics review
Please contact Dr. Heather Ford, if you:
• have questions about your role and rights as a research participant
• wish to obtain more information about clinical research in general
• have concerns, complaints or general questions about the research, or
• wish to provide input about the research study

Statement of Patient Consent
I, __________________________, have been given enough time and opportunity to read and understand the information in this informed consent and ample time and opportunity to ask questions. All my questions have been answered to my satisfaction. I have had sufficient time to consider whether to participate in this study. I understand that my participation in this study is entirely voluntary and that I may withdraw from the study at any time without penalty. The study orthodontist/dentist has my permission to tell my regular doctor about my being in this study:

☐ YES  ☐ NO

I voluntarily consent to participate in this study and will be given a signed copy of this form to take home with me.

Participant's Signature __________________________ Date __________________________
(Signature of participant even if participant is a minor under the age of 18)

(Please also initial each page of this Informed Consent Form)

Parent/Guardian Signature __________________________ Date __________________________
(Parent/Guardian must also sign in addition to participant if participant is under the age of 18)
Faculty of Dentistry
University of Toronto
Discipline of Orthodontics

**Statement of Person Obtaining Consent:**
To the best of my knowledge, the information that I have provided in the response to any questions from the subject, fairly represents the study. I will ensure that the subject receives a copy of this consent form.

Person Obtaining Consent's Signature
Date

**Statement of Study Investigator**
(Investigator preferably to sign the consent form on the same date as the subject, but prior to first patient visit)

I acknowledge my responsibility for the care and well being of the above subject, to respect the rights and wishes of the subject, and to conduct the study in compliance with all the ethical standards that apply to research studies that involve human participants and with applicable Good Clinical Practice guidelines and regulations.

Investigator Name (printed)
Investigator's Signature
Date

*If you have any complaints or concerns about how you have been treated as a research participant, please contact Rachel Zand, Director, Office of Research Ethics, rachel.zand@utoronto.ca or 416-946-3389.*
An initial pilot study was undertaken in order to achieve the following objectives: a) Ensure that the protocol is sound and an adequate volume of collected GCF is measurable, b) Ensure that NO is detectable in the collected GCF, and, c) Serve as a basis for selection of definitive time points for collection of patient samples.

The pilot study was conducted as described previously, where samples of GCF were collected prior to the placement of separators mesial and distal to the maxillary first molars of three male volunteers, and at 1 hour following separator placement (“post-separators”) and at either 3 days or 1 week post-separators.

The preliminary results with the GCF samples demonstrated a promising trend for the early time points. At one hour post-separator placement, 57%, 75%, or 100% of all of the sites sampled for each of the three subjects, respectively, demonstrated an increase in NO levels (Figures 9-11). At 3 days and 1 week post-separators the levels of NO, for the most part, had decreased substantially (Figures 9-11). The decrease observed at 3 days and 1 week post-separators could, however, be due to the fact that the orthodontic force produced by the elastic separators was not continuous and had most likely dissipated.

This preliminary data demonstrated the ability to consistently collect GCF samples of sufficient measurable quantities from buccal and lingual sites of the maxillary molars. In addition, it was also shown that the levels of NO could be reproducibly measured in the GCF samples reliably and consistently. For the main study, the use of fixed appliances (versus elastic separators) for force application creates a more consistent and prolonged force delivery.
**Figure 9:** Total Nitrite levels (μM) at each specific tooth number and site in GCF samples for pilot study participant #1, taken immediately before, 1 hour following, and 1 week following the placement of elastic separators mesial and distal to Teeth #16 and #26. 57% of sites demonstrated an increase in NO levels 1 hour following the placement of separators when compared to the initial baseline reading.

**Figure 10:** Total Nitrite levels (μM) at each specific tooth number and site in GCF samples for pilot study participant #2, taken immediately before, 1 hour following, and 3 days following the placement of elastic separators mesial and distal to Teeth #16 and #26. 75% of sites demonstrated an increase in NO levels 1 hour following the placement of separators.
Figure 11: Total Nitrite levels (μM) at each specific tooth number and site in GCF samples for pilot study participant #3, taken immediately before, 1 hour following, and 3 days following the placement of elastic separators mesial and distal to Teeth #16 and #26. For this subject samples were only taken at the buccal site of each tooth (as opposed to buccal and lingual). 100% of sites demonstrated an increase in NO levels 1 hour following the placement of separators.

APPENDIX D – PARTICIPANT STATISTICS (AGES AND TIME POINTS FOR SAMPLE COLLECTION)

Table 5: Summary of Main Study patient’s ages and time for third sampling time point (three or four days)

<table>
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Mean: 14y 0m 7 pt @ 3d, 6 pts @ 4d
E.1 Saliva: Introduction and Background

A biological fluid that has become increasingly more popular and advantageous for diagnostics, especially in the periodontal disease research field, is saliva. Saliva is a hypotonic fluid composed primarily of water, electrolytes and organic molecules such as amino acids, proteins and lipids (Miller et al., 2010). Saliva (whole or mixed) originates from secretions of the minor and major salivary glands, as well as bronchial and nasal secretions, serum transudate from the mucosa and sites of inflammation, bacteria and their byproducts, viruses, fungi, epithelial and immune cells, GCF, and food debris (Sreebny, 1989). GCF flows into the oral cavity and analysis of mediators in whole saliva has been utilized in many approaches, notably in the development of non-invasive and less time-consuming ways to collect biological fluids for simple diagnostic tests (Lamster and Ahlo, 2007).

The type of saliva that can be sampled is either whole or glandular-specific saliva. The three major salivary glands that introduce saliva into the oral cavity are the parotid, submandibular, and sublingual glands. A mixture of serous- and mucous-derived fluids is produced from these glands, and glandular-specific saliva is primarily used to detect gland-specific pathology (Miller et al., 2010). The glandular composition of whole saliva, in contrast, is listed in Table 6.
Table 6: Percentage contribution of major and minor salivary glands to whole saliva.

(Adapted from (Miller et al., 2010))

<table>
<thead>
<tr>
<th>Salivary Gland</th>
<th>Fluid contribution to whole saliva</th>
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<tbody>
<tr>
<td>Submandibular Gland</td>
<td>65%</td>
</tr>
<tr>
<td>Parotid Gland</td>
<td>23%</td>
</tr>
<tr>
<td>Sublingual Gland</td>
<td>4%</td>
</tr>
<tr>
<td>Minor Salivary Glands</td>
<td>8%</td>
</tr>
</tbody>
</table>

In general, unstimulated whole saliva is most frequently sampled because of its non-invasive nature, rapid stimulation, and ease of sampling. It is most commonly collected by the ‘draining’ method, where the subject’s head is tilted forward and saliva is ‘drooled’ from the mouth into a sterile container. Another common modification to this procedure is having a patient expectorate any accumulated saliva every 30s instead of being ‘drooled’ (Miller et al., 2010). Stimulated saliva is generally collected by means of either masticatory or gustatory stimulation (subjects chew on paraffin, or have sour candy drops placed on the tongue, for example). Stimulated saliva is less appropriate for diagnostic applications, because the water phase of saliva is stimulated, resulting in diluted concentrations of the protein or marker of interest. Additionally, the foreign substances used often modulate the pH of the saliva, which is also unfavorable (Oberg et al., 1982). For these reasons, whole unstimulated saliva is most ideal and appropriate for diagnostic studies.

A patient’s ‘resting’ salivary flow can be influenced by a variety of factors. These factors include: 1) Circadian variation, where unstimulated flow peaks around 5 o’clock pm in most individuals; 2) Light and arousal variations, where if the patient is blindfolded or in a dark room the flow rate falls; 3) Hydration, where a loss in 8% of body water results in a cessation of salivary flow; and 4) Exercise and stress, where a dry mouth is a feature of the sympathetic
‘fright and flight’ response. The mean flow rates of unstimulated whole saliva are in the range of 0.1-0.5 mL/minute (Edgar, 1992).

A multitude of biomarkers have been identified in unstimulated whole saliva samples. In addition to periodontal disease and inflammatory biomarkers, a variety of bone remodelling biomarkers have been identified. It has been found that higher levels of alkaline phosphatase (ALP), an enzyme important in active bone remodeling, were found in pregnant women with periodontitis than those with gingivitis or a healthy periodontium (Kugahara et al., 2008). Additionally, salivary ALP levels were five times higher in saliva from patients with periodontal disease than in controls (Totan et al., 2006). It was also found that salivary RANKL levels are significantly higher in periodontitis patients than in patients receiving maintenance therapy (Buduneli et al., 2008). However, studies regarding correlations between salivary levels of osteoprotegerin (OPG) and periodontitis have been contradictory (Buduneli et al., 2008; Miller et al., 2006). Overall, a common goal among these studies is to implement routine regimens in healthcare utilizing saliva samples for widespread screening for bone diseases. This concept reinforces the usefulness of saliva as a convenient, non-invasive, yet useful biological fluid sample. The purpose of this “subsection” of the project was to investigate whether salivary concentrations of NO increased after the application of an orthodontic force.

**E.2 Whole Unstimulated Saliva Collection**

Concurrent with the time points of GCF collection as part of the ‘main’ study, whole unstimulated saliva was also collected at T0, T1, and T2. The method was adapted from Miller et al. (2010). Briefly, patients were asked to refrain from having anything to eat or drink prior to sampling. Participants would rinse their mouth with water, and from a seated position would
expectorate any accumulated saliva into a sterile container every 30s until an adequate volume (approximately 2 mL) was obtained. The saliva samples were stored at -30ºC until the day of laboratory analysis.

E.3 Laboratory Analysis Protocol: Salivary Nitrite Levels

The saliva samples were plated in triplicate on 96-well plates as previously described in section 3.4.1 without dilution in PBS, and nitrite concentration was measured with the Griess Reagent System as described in section 3.4.2.

E.4 Results of Salivary Nitrite Levels at T0, T1, T2

The nitrite concentration (μmol/L) in the saliva of each patient at each time point was calculated. The mean values and standard deviations are listed in Table 7. Table 8 lists the comparative statistics for nitrite concentrations in the whole unstimulated saliva samples, where it was found that a statistically significant decrease in salivary nitrite concentration was found between T0 and T1, and between T0 and T2. Figure 12 depicts these values graphically.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Mean (sd)</th>
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<tr>
<td>Baseline (T0)</td>
<td>132.7 (73.8)</td>
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<tr>
<td>T1 (1 hour)</td>
<td>77.9 (43.8)</td>
</tr>
<tr>
<td>T2 (3-4d)</td>
<td>86.4 (36.6)</td>
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</table>

<table>
<thead>
<tr>
<th>Salivary Samples</th>
<th>T0 vs. T1</th>
<th>T0 vs. T2</th>
<th>T1 vs. T2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.00244*</td>
<td>0.0256*</td>
<td>0.3125</td>
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</table>

Table 7: Mean Nitrite Concentrations in Whole Unstimulated Saliva Samples (μmol/L)

Table 8: Comparative Statistics (P-Values)
Figure 12: Mean whole unstimulated salivary nitrite concentrations (µmol/L) at baseline (T0), T1 (1 hour), T2 (3-4 days). The middle line in the box plot represents the mean value, and the upper and lower portions of the box represent the 75th and 25th percentiles. A significant decrease was found between T0 and T1, and T0 and T2 ($p<0.05$).

E.5 Analysis of Salivary Results

A significant decrease in mean salivary nitrite concentrations was observed between T0 and T1, and T0 and T2. The significant decrease that was noted at 1 hour post-bonding (T1) is attributed to the dehydrating nature of the orthodontic bonding procedures. As the braces are bonded, the oral cavity is isolated to prevent salivary contamination onto the teeth. This involves placement of gauze and cotton rolls, as well as frequent rinsing with water. These processes are proposed to be the reason that a decrease in nitrite concentration was noted – essentially the sample became dilute due to the dehydration and rehydration with water for rinsing.

The significant decrease in salivary nitrite concentrations that was observed between T0 and T2 (3-4 days later) was a more interesting finding. It is unclear as to why this finding was seen; however, several hypotheses are proposed. The first hypothesis is that the circadian changes in salivary flow rate played a role in the dilution of the saliva samples. Briefly, Edgar (1992) reported that peak salivary flow rates were recorded to be around 5 o’clock in the
evening. Due to schedule restrictions at the Faculty of Dentistry, T0 sampling for most patients took place at the beginning of scheduled clinic sessions, typically 9 am or 2 pm. When patients returned 3-4 days later, 78.9% of them returned around 4-4:30pm, closer to when the peak salivary flow rate is at its highest. A higher salivary flow rate produces increased water content in the saliva, which then dilutes the nitrite levels in the saliva samples (Edgar, 1992).

Another hypothesis to explain the statistically significant decrease in salivary nitrite concentration that was observed between T0 and T2 was reported by Jin et al. (2013). This study reported that salivary nitrite concentrations in rats and in humans increased to a significant degree when the humans or rats were under stress. Salivary nitrite concentrations decreased when the stressful situation was removed. It is possible that patients become more anxious on the day they are to receive braces than when they return 3-4 days later. It is not clear whether patient’s stress levels truly played a role; however, the possibility remains that it may.

Finally, the Griess Reagent system is a colorimetric assay. Although its effectiveness has been proven in GCF and salivary research, when saliva is utilized as the sampling fluid, there are variations in density of each saliva sample. Although previous studies would argue against this concept, it is not completely clear if variations in salivary density played a role in the absorbance values that were read with each sample. If there was an effect, this would create significant variations in the salivary sample readings, and decrease the reliability of this test. Further investigation into the reliability of the Griess test for use in saliva, as well as further investigation into salivary nitrite levels is warranted.