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DOES MENOPAUSE AFFECT CYTOKINE RESPONSE TO ORTHODONTIC THERAPY?

by

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DEDICATION

I dedicate this work to my family, which has been instrumental in my development and education. I also dedicate this thesis to my fiancée, Juliann, whose love and encouragement supported me to the finish.

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ABSTRACT

Does menopause affect cytokine response to orthodontic therapy?

by

Corey Steven Jones

The objective of this study was to elucidate the cytokine response in pre-vs post-menopausal adult women receiving orthodontic treatment. Thirty (30) women were initially enrolled to participate in this IRB approved study: 16 pre-menopausal and 14 post-menopausal. Patients were excluded based on self-report of use of anti-inflammatory medications/supplements in the past 30 days or self-report of any known conditions that would modulate systemic inflammation. Patients were also required to be at least 2 months into active orthodontic therapy, and had to present with and absence of periodontal inflammation. Patients were considered to be pre-menopausal (control) if they were still experiencing regular menstrual cycles or post-menopausal (experimental) if they had not menstruated in the past 12 months.

GCF samples were obtained for each participant prior to orthodontic activation (T_0) of the dentition; GCF samples were re-collected 24 hours later (T_1) from the same sites. A Luminex assay was completed to analyze the concentrations of GCF IL-1β, IL-6, and TNF-α.

All subjects, regardless of pre- vs. post-menopausal status showed a significant increase from T_0 to T_1 for [IL-1β], [IL-6], and [TNF-α] ($p < 0.05$). No significant difference was noted at T_0 in [IL-1β] for post-menopausal compared to pre-menopausal participants. However, there was a significant increase in GCF [IL-1β] in post-menopausal participants compared to pre-menopausal participants after activation at T_1 ($p < 0.05$). Additionally, there was a significant increase in GCF [IL-1β] in post-menopausal participants compared to pre-menopausal participants for ΔT_1-T_0 ($p = 0.05$). There were no statistically significant differences noted for T_0, T_1, or ΔT_1-T_0 for [IL-6] or [TNF-α] when comparing pre- vs. post-menopausal groups.

We conclude that GCF concentration of cytokines IL-1β, IL-6, and TNF-α increase after orthodontic activation in pre- and post-menopausal females. Post-menopausal females with a healthy periodontium show a significantly increased inflammatory response for [IL-1β] compared to the pre-menopausal subject females. There is not a statistically significant difference in GCF IL-6 and TNF-α cytokine response in pre- and post- menopausal women receiving orthodontic treatment.

LITERATURE REVIEW

Menopause is the time in a woman's life when ovarian estrogen production ceases, and the body, in turn, experiences a variety of systemic changes. Estrogen is a key bone turnover regulator (Riggs 1986). As women age and estrogen production decreases, bone's balance between deposition and resorption is disturbed; the result is a net loss of bone (Heaney 2003, Garnero et al. 1999, and Garnero et al. 1996). Additionally, Recker et al. (2004) found that bone-remodeling rates doubled at menopause ($p < 0.001$) and tripled 13 years after menopause ($P < 0.008$), which contributed to increased age-related skeletal fragility in women. Thus, women experience increased remodeling as they age, with more resorption occurring compared to apposition, and this confers increased risk for skeletal fragility. This excessive resorption and/or osteoporotic progression can be debilitating and represents one of the major health and socio-economic problems of modern society (Payne 1997).

Bone tissue is a living organ, which forms in one of two different ways: endochondral or intramembranous formation. In the endochondral model, a cartilage framework forms first, which later mineralizes into bone tissue (e.g. long bones). With intramembranous bone, however, rudimentary bone tissue is initially created with no cartilage intermediary (e.g. alveolar bone). This rudimentary tissue will

continue to mature and mineralize into a stronger, more functional form. However, as a living tissue, bone is not static after reaching its mature state; instead bone continuously remodels both during development of the skeleton and throughout adulthood. The basis for the remodeling process is rooted in the coupled interplay between bone-forming osteoblasts and bone-resorbing osteoclasts (Lerner 2006).

Remodeling occurs in localized groups of osteoblasts and osteoclasts known as basic multicellular units (BMUs). Each BMU is comprised of a "cutting cone" of osteoclasts, used to resorb bone, followed by trail of osteoblasts, which fill the bony defect left by osteoclasts (Hardy and Cooper 2009). Remodeling in bone multi-cellular units starts with osteoclastic resorption; however, osteoclast formation and activation are controlled by osteoblasts, and as such, the true beginning of remodeling involves catabolic activation of osteoblasts. It remains unknown which molecules activate this change in the phenotype of osteoblasts, but it is known that physiological processes, such as loading and hormonal regulation of calcium homeostasis, play a crucial role. This activation initially leads to osteoblastic degradation of the unmineralized osteoid that exists between the osteoblastic cell layer and the mineralized bone. This step is necessary since osteoclasts cannot adhere to unmineralized bone; they are only capable of resorbing mineralized bone (Lerner 2006). Next, the osteoblasts increase expression of receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF). Simultaneously, expression of osteoprotegerin (OPG), a competitive inhibitor of RANKL, is decreased. RANKL then binds the receptor activator of nuclear factor κB

(RANK) on osteoclast progenitor cells, which leads to further development and activation of osteoclasts. This process is occurring concurrently with M-CSF binding of receptor c-Fms, which will lead to an increase of the osteoclast progenitor pool, increased survival of said cells, and further differentiation of mononucleated progenitor cells into multinucleated (Lerner 2006). Osteoclast activation is influenced by a number of systemic and local regulators, as described later in this review. The final step in activation of the remodeling process is the retraction of the osteoblasts from the bone surface, so that the activated multi-nucleated osteoclasts can access mineralized bone – the demineralized organic bone matrix will subsequently be degraded by proteolytic enzymes. Subsequently, osteoclasts create resorption lacunae, and "less-well-organized" mononuclear cells appear in the lacunae to clean up the organic matrix left behind by osteoclasts. Thereafter, osteoblast precursor cells are recruited to the lacunae, where they differentiate into fully active osteoblasts that fill the resorption lacunae with new bone. Given all the above, perhaps the most crucial event in the initiation of bone remodeling is the activation of receptors RANK and c-Fms on osteoclast precursor cells. And, it has now been well documented that the regulation of RANKL/RANK/OPG system, as well as M-CSF expression, is highly influenced by and interdependent upon systemic hormones and local inflammatory cytokines (Lerner 2006).

Parathyroid hormone (PTH) is an important systemic regulator of bone remodeling and calcium homeostasis. PTH stimulates bone resorption, increases renal tubular calcium reabsorption, and increases renal Calcitriol (active Vitamin D)

production. Calcitonin functions to antagonize the effects of PTH; Calcitonin inhibits the secretion of osteoclast proteolytic enzymes, minimizes osteoclast motility, and modifies the morphologic shape of osteoclasts themselves (Hadjidakis and Androulakis 2006). Growth hormone affects adult bone mass via regulation of both bone formation and bone resorption (Hadjidakis and Androulakis 2006, Wang et al. 2004). In addition, glucocorticoids exert both stimulatory and inhibitory effects on bone cells; they can promote osteoblast differentiation and sensitize bone cells to regulators of bone remodeling, as well as augment osteoclast recruitment (Hadjidakis and Androulakis 2006, Weinstein et al. 1998). Thyroid hormone also stimulates both bone resorption and formation, and a hyperthyroid patient who experiences excessive bone turnover is at an increased risk of bone loss (Hadjidakis and Androulakis 2006, Britto et al. 1994). Estrogen, which is significantly decreased in post-menopausal females, helps battle bone resorption. Estrogen hormones decrease the responsiveness of osteoclasts to RANKL and decrease the RANKL/OPG ratio (expanded upon below), thereby preventing osteoclast formation. Furthermore, estrogen stimulates osteoblast proliferation and decreases their apoptosis (Hadjidakis and Androulakis 2006, Kameda et al. 1997).

Local signaling molecules also influence bone remodeling; cytokines are one such molecule (Uematsu et al. 1996, Horowitz et al. 1989). The first demonstration that immune-competent cells could influence bone remodeling was in an in-vitro study by Horton et al. in 1972. This team found that "osteoclast-activating factor" could be used to stimulate bone resorption when placed in contact with dental plaque.

"Osteoclast-activating factor" was purified 13 years later (Dewhirst et al. 1985), and the resulting purified form is what we know today as interleukin-1β (IL-1β). Shortly after, it was also shown that cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were also able to stimulate osteoclast formation and bone resorption (Lerner 2006, Bertolini et al. 1986, and Ishimi et al. 1990). Moreover, IL-1β, TNF-α, and IL-6 have been found at increased levels in cases of inflamed gingiva and periodontitis (Lerner 2006). These same cytokines are also highly expressed in synovial fluid from patients with rheumatoid arthritis, and have been also implicated in the pathogenetic mechanisms that lead to excessive bone resorption from inflammatory diseases, such as osteoporosis (Lerner 2006, Choy and Panayi 2001). Thus, the same types of cytokines have been implicated as stimulators of bone resorption in periodontitis, rheumatoid arthritis, and post-menopausal osteoporosis (Lerner 2006).

The cellular sources responsible for the production of inflammatory cytokines are varied. According to Graves and Cochrane (2003), the following cells produce IL-1β and TNF-α: monocytes and macrophages, polymorphonuclear leukocytes, fibroblasts (gingival and PDL), epithelial cells, endothelial cells, and osteoblasts. These cells are all components of the body's inflammatory system, and as such, they play a role in orthodontic tooth movement. Davidovitch et al. (1988) found that early orthodontic tooth movement involves an acute inflammatory response, in which leukocytes migrate out of the PDL, and interact directly with osteoblasts and osteoclasts, or indirectly with neighboring monocytes/macrophages, lymphocytes,

and fibroblasts. This again confirms that the same inflammatory components that may be found in a pathological resorption setting like periodontitis are also at play during non-pathological resorption, vis-a-vis orthodontic tooth movement.

Interleukin-1β and TNF-α behave synergistically to facilitate local bone resorption (Graves and Cochrane 2003), and IL-1β is also considered to be a powerful inducer of IL-6 production (Linkhart et al. 1991, Uematsu et al. 1996). Interleukin-6 secreted by osteoblasts, osteoclasts, and stromal cells promotes further osteoblast production in conditions of high bone turnover (Sims et al. 2004). Likewise, IL-1β, IL-6, TNF-α, and Epidermal Growth Factor (EGF) all overlap with similar functions to facilitate bone resorption (Uematsu et al. 1996). These aforementioned mutual cytokines also interact with other key molecules necessary for bone remodeling - the receptor activator of nuclear factor κB (RANK) and its associated ligand RANKL.

The interactions between the inflammatory cytokines and the RANK/RANKL system have also been well documented. Interleukin-1β and TNF-α act synergistically with RANKL to enhance bone resorption. TNF-α can also stimulate osteoclast formation in the absence of stromal cells/osteoblasts if a "constitutive level" of RANKL (i.e. a level not leading to stimulation of osteoclast formation by itself) is present (Lerner 2006, Lam et al. 2000). This relationship is not surprising, however, since both TNF-α and RANKL are both members of the TNF ligand superfamily. Wei et al. (2005) showed that TNF-α enhances RANKL expression in stromal cells, due mainly because of the induction of IL-1 receptors. Furthermore, they found that activation of TNF-α receptors in osteoclast progenitor cells leads to enhanced

expression of RANK, IL-1 stimulatory receptors, and decreased expression of inhibitory IL-1 type II receptors. Interleulkin-1β also enhances the osteoclastogenic effect of TNF-α by increasing stromal cell expression of RANKL and directly stimulating osteoclast differentiation.

In women specifically, the net loss of bone incurred during remodeling is made worse by an increased remodeling frequency that occurs after menopause (Garnero et al. 1999, Garnero et al. 1996). In other words, not only do post-menopausal osteoblasts have a decreased ability to fill the resorption lacunae, but also, they are being more frequently activated to do so. This imbalance in bone remodeling, which favors loss of bone tissue, can also lead to other pathologies, such as osteoporosis, rheumatoid arthritis, periodontitis, osteomyelitis, and lost teeth. Increased frequency of bone multicellular unit (BMU) activation in post-menopausal women leads to increased numbers of osteoclasts and resorption lacunae in the skeleton (Lerner 2006). However, even with increased activity of osteoclasts, patients typically do not exhibit increased serum calcium levels, due to the tight control from the body's calcium-regulating hormones. Yet, the excretion of calcium in urine *does* increase, along with deoxypyridinoline crosslinks - which comes as a consequence of increased degradation of the abundant type I collagen fibers in the bone extracellular matrix (Lerner 2006). Since the number of bone-resorption sites is increased, it naturally follows that the number of bone-formation sites be increased concordantly. Osteocalcin, which is secreted by osteoblasts, is a commonly used serum marker for bone formation. In post-menopausal osteoporosis, osteocalcin

levels are increased, not because individual osteoblasts make more osteocalcin, but rather because the increased activation frequency of osteoblasts in BMUs (Lerner 2006). Additional serum bone formation markers include alkaline phosphatase and procollagen I carboxy-terminal extension peptides. Markers of bone resorption are found in plasma and urine: plasma markers include tartrate-resistant acid phosphatase and pyridinoline; urinary markers of resorption include pyridinoline and deoxypyridinoline (collagen cross-link) containing peptides (Jeffcoat 1998).

The cause of post-menopausal bone loss is multifactorial; bone loss is due to both direct and interrelated effects of inflammation, poor nutrition, reduced body mass, immobility, and side effects of medical treatment, such as glucocorticoid therapy (Hardy and Cooper 2009). The pathologic mechanisms behind these factors are complex, but ultimately, their effects are mediated through the inflammatory system. As noted prior, one particularly impactful causative factor is the decreased estrogen levels that occur in females after menopause. Estrogen has been shown to decrease bone-resorbing activity and enhance apoptosis of differentiated osteoclast cells (Taranta et al. 2002, Lerner 2006). Activation of estrogen receptors on osteoclasts can also lead to inhibition of osteoclast formation by interrupting both the M-CSF and RANKL activation at their respective receptors, c-Fms and RANK (Shevde et al. 2000, Sarma et al. 1998, Lerner 2006). Estrogen has an effect on osteoblasts, as well. Estrogen has been demonstrated to increase OPG production in human osteoblast cells (Hofbauer et al. 1999, Lerner 2006); OPG then goes on to act as a competitive inhibitor of RANKL, and decreases osteoclast differentiation. As

described above, the RANK/RANKL/OPG system is often influenced by other cytokines, such as IL-1β, IL-6, and TNF-α. Not surprisingly, estrogen can affect these cytokines, as well. Estrogen is a potent inhibitor or IL-1β and TNF-α production in bone marrow cells and monocytes (Pacifici 1999, Lerner 2006); estrogen also interrupts the production of IL-6 in stromal cells and osteoblasts (Girasole et al. 1992). Given the above benefits of estrogen, it is clear to see why a lack of estrogen can have damaging consequences for bone resorption. Without estrogen acting as a check against excessive resorption, the elevated remodeling rates and tipped balance toward bone resorption leads to increased bone-loss for post-menopausal females.

Inflammatory markers can provide an indication of local remodeling activity. Studies of inflammatory cytokines can be designed to obtain these localized samples in alveolar bone – a weakness associated with studies using previously mentioned variables. Gümüş et al. (2013) compared females (age 25-68) with rheumatoid arthritis or osteoporosis to systemically healthy females – all of whom had periodontal disease. By sampling gingival crevicular fluid (GCF) and patient serum, this team found that patients with rheumatoid arthritis had significantly higher levels of RANK/RANKL and Interleukin-17A (IL-17A) than the osteoporotic and systemically healthy patients. However, there were some trends that showed increased overall levels of IL-17 and RANK/RANKL of osteoporosis patients when compared to systemically health patients. One drawback of this study, however, is that every subject presented with periodontitis, since inflammation caused by

periodontitis confounds the true picture of how inflammation regulates bone turnover in post-menopausal subjects.

Other studies have tested alveolar bone for inflammatory GCF cytokine levels in non-pathologic settings; one dental specialty particularly tailored for such an investigation is orthodontics. Orthodontic tooth movement (OTM) utilizes the body's inflammatory and associated bone remodeling system to move teeth through alveolar bone. Not surprisingly, the body uses the same inflammatory cascade and machinery to facilitate bone remodeling and tooth movement. However, in the case of OTM, the inflammatory response is being initiated by a non-pathologic cause (periodontal ligament distortion), whereas the processes mentioned above were prompted by autoimmune or infectious reasons.

The commonly accepted theory behind OTM is known as the pressure-tension theory. When a tooth is moved within its socket (with light pressure), blood vessels within the PDL become partially compressed on the pressure side and dilated on the tension side; concurrently, the PDL fibers and cells are mechanically distorted (Masella and Meister, 2006). Within minutes of these changes, cytokines and prostaglandins are released, and within a few hours, the metabolic effects of said chemical messengers affect cellular activity. As early as four hours after PDL distortion, cells can differentiate from precursors into osteoclasts and osteoblasts, and in two days, tooth movement begins as osteoclasts/osteoblasts remodel the bony socket. This cascade of events arises from the difference in pressure (or compression) versus tension affecting the periodontal ligament (Masella and Meister 2006).

Clinical evidence of this phenomenon has been well documented. In 1996, Uematsu et al. examined the levels of IL-1β, IL-6, and TNF-α in a split mouth study during canine retraction. This team measured inflammatory cytokine (listed previously) levels in GCF at baseline, 1 hour, 24 hours, and 168 hours after activation canine retraction. They found increased levels of IL-1β, IL-6, and TNF-α at each time point, with the maximum level found at 24 hours later ($P < .05$). This pattern was not found at the control site. Strengths of this study are that it controlled for any corruption of data, by eliminating the confounding inflammation caused by poor hygiene, and eliminated any reduction of the inflammatory response that would be caused by anti-inflammatory drugs.

In 2011, Faulkner analyzed GCF volumes and contents for patients undergoing orthodontic treatment. GCF cytokines were measured prior to activation and at 1, 6, and 24 hours post activation. The same protocol was followed for control teeth, which were not bonded throughout the duration of the study. Data collection continued 5-7 weeks later, where control and experimental teeth were sampled in the same manner. Total protein levels ranged from 3 μg to 85 μg in the control samples, whereas the experimental GCF total protein levels ranged from 4 μg to 175 μg. The mean concentration of GCF IL-6 for the MB of experimental teeth (83.85 fg/μg) was significantly higher than the mean GCF IL-6 protein for control teeth (50.67 fg/μg). In addition, the maximum concentration of IL-6 in the GCF was found to be 6 hours and 24 hours after activation. However, the mean GCF IL-6 protein at the DL of experimental teeth was only 49.10 fg/μg; this information, coupled with large

standard deviations and a small sample size limit the power behind these results.

Grieve III et al. (1994) completed a similar study wherein they examined the GCF levels of Prostaglandin E (PGE) and IL-1β. The study included 10 patients, each having one treatment tooth being compared to one unbonded control, contralateral tooth. In this study, GCF was sampled at the *compression* sites before activation, then samples were collected again at 1 hour, 24 hours, 48 hours, and 168 hours later. The inclusion criteria also required generalized probing depths of $\leq$ 3 mm and no radiographic evidence of bone loss, to minimize the influence of plaque-induced inflammation and isolate the study of mechanically stimulated PGE and IL-1β. At 1 hour and 24 hours after activation, the mean GCF IL-1β levels were significantly elevated at treatment teeth (8.9 $\pm$ 2.0 and 19.2 $\pm$ 6.0 pg, respectively) compared with control teeth (2.0 $\pm$ 1.1 pg and 2.9 $\pm$ 1.0 pg, respectively). Again, the greatest, significant increase in GCF IL-1β was found at 24 hours post-activation. Thus, it has been shown repeatedly that cytokine levels elevate after orthodontic activation, as predicted by the pressure-tension theory, and this increase can be measured via GCF collection.

Ren and Vissink (2008) completed a review to further evaluate cytokines in the GCF during orthodontic tooth movement. After analyzing the 23 studies that fit the inclusion criteria, this review found that cytokines predominantly peaked 24 hours after activation, irrespective of treatment mechanics. They also found that without force re-activation, inflammatory cytokines decrease back down to baseline levels 168 hours after orthodontic activation. In addition, three studies compared cytokine

levels at pressure versus tension sides, only one of which reported a significant difference (higher cytokine levels at the tension side). Due to the small amount of research in this area, no conclusions can be drawn about the difference (or lack thereof) of the inflammatory response on the tension versus compression side of a tooth undergoing OTM. Due to significant heterogeneity in the studies reviewed, a full meta-analysis could not be completed.

Women today are living longer, working longer, and spending more money on their appearance while doing so (Schlessinger et al. 2010). Not surprisingly, orthodontics is seeing the effects this trend, as the number of adult, esthetic-conscious female patients has also increased. This, in turn, creates more opportunity for orthodontic treatment and practice growth. However, as this is a new and growing patient demographic, it is the orthodontist's responsibility to understand the biology and physiology of this group.

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STATEMENT OF THE THESIS

It is the orthodontist's responsibility to understand the biology and physiology of treatment. The number of adult female orthodontic patients continues to rise, but this demographic remains largely understudied – particularly, post-menopausal females. The purpose of this study is to elucidate GCF inflammatory cytokine response in pre- and post- menopausal women receiving orthodontic treatment.

MANUSCRIPT

ABSTRACT

Title: Does menopause affect cytokine response in orthodontic therapy?

Introduction: Post-menopausal hormonal changes alter the female body's equilibrium between bone deposition and resorption, with a net increase in resorption. The inflammatory cytokines contributing to the net loss in bone are also at work during orthodontic tooth movement. The aim of this study was to elucidate the cytokine response in pre-vs. post-menopausal adult women receiving orthodontic treatment. **Methods:** Thirty (30) women were enrolled in the study and separated into 2 groups: pre- (16) and post-menopausal (14). Cytokine activity was evaluated by sampling gingival crevicular fluid (GCF) immediately before (T_0) and 24-hours after (T_1) orthodontic appliance activation. GCF cytokine concentrations (pg/mL) for interleukin-1 β (IL-1β), interleukin-6 (IL-6), and Tumor Necrosis Factor-α (TNF-α) were compared at T_0 and T_1 between the two groups. **Results**: Both the pre-and post-menopausal groups showed significant increases from T_0 to T_1 for IL-1β, IL-6, and TNF-α (IL-1β pre-meno T_0 7.99± 7.86 vs. T_1 12.66± 6.34, P< 0.0005; post-meno T_0 10.56± 7.69 vs. T_1 23.38± 17.53, P< 0.0082; IL-6 pre-meno T_0 1.88± 1.18 vs. T_1 3.39± 1.33, P< 0.0002; post-meno T_0 1.74± 0.92 vs. T_1 3.22± 1.64, P< 0.002; TNF-α pre-meno T_0 2.78± 1.51 vs. T_1 5.37± 3.2, P< 0.005; post-meno T_0 2.2± 0.64 vs. T_1 4.5± 3.47, P< 0.01). There was a significant difference in [IL-1 β] for pre-menopausal participants compared to post-menopausal at T_1 (12.66± 6.34 vs. 23.38± 17.53, p< 0.039), and a significant difference in GCF [IL-1 β] response in pre-menopausal participants compared to post-menopausal for ΔT_1-T_0 (5.87± 4.13 vs. 14.86± 14.69, P= 0.05). There were no significant differences noted for [IL-6] or [TNF-α] when comparing pre- vs. post-menopausal groups. **Conclusions:** Post-menopausal females show a significantly increased inflammatory response for IL-1β compared to the pre-menopausal subject females.

Does Menopause Affect Cytokine Response in Orthodontic Therapy?

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INTRODUCTION

A majority of patients seeking cosmetic therapy are adult females, often mothers, who are more educated and career-oriented compared to previous generations.[1] In addition, as women age, they are often concerned with the aspects of treatment concerning the lower half of the face.[2] However, another characteristic associated with aging women is decreased estrogen production. Ovarian cessation of estrogen production at menopause effects skeletal bone regulation, which results in excessive resorption and predisposition for osteoporosis,[3-7] a major health and socio-economic problem.[8]

The physiologic foundation for bone turnover is rooted in the coupled interplay between bone-forming osteoblasts and bone-resorbing osteoclasts.[9] Estrogen-deficient females show higher serum levels of the cytokines known to upregulate osteoclastic resorption in the skeleton.[10-12] These cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6), and Tumor Necrosis Factor-α (TNF-α), play an integral role in the local regulation of osteoblasts and osteoclasts.[13-20] They exert an influence both on osteoblasts and osteoclasts directly and indirectly through the RANK Ligand, IL-1β, and TNF-α interaction to enhance local resorption.[21] Interleukin-1β is also considered to be a powerful inducer of IL-6 production.[13,22] In addition, Interleukin-6 secreted by osteoblasts, osteoclasts, and stromal cells promotes further osteoblast production in conditions of high bone turnover; however, IL-6 is also known to contribute to *both* pro-inflammatory and anti-inflammatory cascades.[23]

Orthodontic tooth movement generates an inflammatory process. Alveolar bone remodeling is at work during orthodontic tooth movement (OTM), through the commonly accepted pressure-tension theory,[16] whereby blood vessels within the PDL become partially compressed on the pressure side and dilated on the tension side. The PDL fibers and cells are mechanically distorted. Within minutes of these changes, cytokines are released, and within a few hours, the metabolic effects of said chemical messengers affect cellular activity.[16] As early as four hours after PDL distortion, cells can differentiate from precursors into osteoclasts and osteoblasts, and in two days, tooth movement begins as osteoclasts/osteoblasts remodel the bony socket.[16]

Clinical evidence shows elevated inflammatory cytokines during orthodontic tooth movement. In 1996, Uematsu et al.[13] found increased levels of IL-1β, IL-6, and TNF-α in a split mouth study during canine retraction, with the maximum level found at 24 hours post-activation.

It is the orthodontist's responsibility to understand the biology and physiology behind treatment. The number of adult female orthodontic patients continues to rise, but this demographic remains largely understudied – particularly, post-menopausal females. The purpose of this study is to elucidate GCF inflammatory cytokine response in pre- and post- menopausal women receiving orthodontic treatment.

MATERIALS AND METHODS

Participants

Sixty-one (61) participants were screened at the CWRU Orthodontic Clinic and from private practices located in Northfield, OH, Hudson, OH, and Avon Lake, OH. The Institutional Review Board approved this prospective case control study (approval no 2012-196). Thirty (30) subject met the inclusion criteria (Table I) and consented to participate. Participants were considered post-menopausal if they reported no menstruation in the last 12 months.

Data Collection

GCF was obtained from the control and experimental subjects just prior to orthodontic activation of the dentition (T_0). Orthodontic activation was defined as placing new Invisalign™ trays or ligating a metal wire with full engagement into the bracket slot. GCF samples were recollected 24 hours later (T_1) for each subject at the identical teeth. Probing was completed prior to GCF collection to ensure no BOP. Plaque and debris were removed from the tooth with gauze; the area was isolated with air and cotton rolls. All GCF sample collection was completed by two investigators (CJ and SS); Periopaper™ (Harco) strips were used for GCF collection. Each strip was inserted into the sulci until saturation of the apical 1/3 of the strip. Four strips were used to collect GCF fluid from each patient – 2 strips in the anterior and 2 in the posterior. All 4 strips were combined and stored in a sterile polypropylene vial and considered to be one sample; this "pooled sample" provides an overall patient-centered response rather than a site-specific response. The samples were initially placed in a dry ice cooler (sublimating temp. dry ice is -109°C – -79°C). The samples were then transferred into a larger storage freezer, which maintained a constant -80°C

temperature. Once all samples had been collected, they were transferred via dry ice packaging to the lab for processing and analysis.

A multiplex kit was purchased for IL-1β, IL-6, and TNF-α from the Luminex Corporation (Austin, TX). GCF samples from each subject were eluted in 1mL of phosphate-buffered saline containing 0.05% polysorbate surfactant with a 60-minute incubation on a rotary mixer at +4°C. The multiplex was carried out on 50-µL samples of GCF in duplicate according to the manufacturers' instructions. Well plates were pre-coated with appropriate antibodies, and samples and the standards were added to wells. The detection antibody was then added and incubated at 4°C for 2 hours at room temperature for the TNF-α, IL-1β, and IL-6 assays. After washing, conjugated antibody was added and incubated for 60-120 minutes at room temperature in the dark. Finally, the optical densities were read at 450 nm with a background subtraction at 570 nm, and the samples were compared to the standards.

Data Analysis

A coefficient of determination (R^2) was initially run to determine how well the data fit the statistical ideal model. Data from the thirty participants were entered into an Excel™ (Microsoft Corporation – Redmond, WA) spreadsheet and statistical functions were completed with Excel™ and SPSS™ statistical software (SPSS Inc. – Chicago, IL). Means and standard deviations were calculated for the control and experimental groups' T_0, T_1, and ΔT_1-T_0 values; all values were recorded in pg/mL for each cytokine tested. Shapiro-Wilk tests were used to evaluate the distribution of the data; since 8 out of the 12 groups were classified as normally distributed, we

elected to use parametric statistical tests. In addition, the use of parametric tests allowed for comparison to similar articles that also utilized parametric tests to analyze samples of similar size. T-tests were used to compare mean inflammatory marker values within and between the pre-menopausal and post-menopausal groups. Significance accepted for analysis was set at $p < 0.05$.

RESULTS

IL-1 β

The R^2 coefficient for IL-1β was 0.97 (Table III), which indicates that lab tests are accurate and reliable to standard curves. The mean [IL-1β] for pre-menopausal subjects at T_0 was 7.99 + 7.86 pg/mL, and the mean [IL-1β] for pre-menopausal subjects at T_1 was 12.66 + 6.34 pg/mL (Table IV and Fig 1). The increase between mean T_0 and T_1 [IL-1β] for pre-menopausal subjects was statistically significant ($p < 0.05$, Table IV). The mean [IL-1β] for post-menopausal subjects at T_0 was 10.56 + 7.69 pg/mL, and the mean [IL-1β] for post-menopausal subjects at T_1 was 23.38 + 17.53 pg/mL (Table IV and Fig 1). The increase between mean T_0 and T_1 [IL-1β] for post-menopausal subjects was statistically significant ($p < 0.05$, Table IV).

Table 7 shows the results of t-tests comparing mean [IL-1β] in pre- vs. post-menopausal subjects. The mean T_0 [IL-1β] for pre-menopausal subjects was not significantly different compared to post-menopausal subjects ($P = 0.26$). However, the mean T_1 [IL-1β] for pre-menopausal subjects was significantly smaller compared

to post-menopausal subjects ($P < 0.05$). The difference in mean cytokine response (e.g., ΔT_1-T_0) for [IL-1β] in pre-menopausal subjects was significantly smaller compared to post-menopausal subjects ($P = 0.05$).

IL-6

The R^2 coefficient for IL-6 was 0.98 (Table III), which indicates that lab tests are accurate and reliable to standard curves. The mean [IL-6] for pre-menopausal subjects at T_0 was 1.88 + 1.18 pg/mL, and the mean [IL-6] for pre-menopausal subjects at T_1 was 3.39 + 1.33 pg/mL (Table V and Fig 2). The increase between mean T_0 and T_1 [IL-6] for pre-menopausal subjects was statistically significant ($p < 0.05$, table 5). The mean [IL-6] for post-menopausal subjects at T_0 was 1.74 + 0.92 pg/mL, and the mean [IL-6] for post-menopausal subjects at T_1 was 3.22 + 1.64 pg/mL (Table V and Fig 2). The increase between mean T_0 and T_1 [IL-6] for post-menopausal subjects was statistically significant ($p < 0.05$, Table V).

Table 7 shows the results of t-tests comparing mean [IL-6] in pre- vs. post-menopausal subjects. The mean T_0 [IL-6] for pre-menopausal subjects was not significantly different compared to post-menopausal subjects ($P = 0.64$). The mean T_1 [IL-6] for pre-menopausal subjects was not significantly different compared to post-menopausal subjects ($P = 0.98$). The difference in mean cytokine response (e.g., ΔT_1-T_0) for [IL-6] in pre-menopausal subjects compared to post-menopausal subjects was not statistically significant ($P = 0.60$).

TNF-α

The R^2 coefficient for TNF-α was 0.96 (Table III), which again indicates that lab tests are accurate and reliable to standard curves. The mean [TNF-α] for pre-menopausal subjects at T_0 was 2.78 + 1.51 pg/mL, and the mean [TNF-α] for pre-menopausal subjects at T_1 was 5.37 + 3.20 pg/mL (Table VI and Fig 3). The increase between mean T_0 and T_1 [TNF-α] for pre-menopausal subjects was statistically significant ($p < 0.05$, Table VI). The mean [TNF-α] for post-menopausal subjects at T_0 was 2.20 + 0.64 pg/mL, and the mean [TNF-α] for post-menopausal subjects at T_1 was 4.50 + 3.47 pg/mL (Table VI and Fig 3). The increase between mean T_0 and T_1 [TNF-α] for post-menopausal subjects was statistically significant ($p < 0.05$, Table VI).

Table 7 shows the results of t-tests comparing mean [TNF-α] in pre- vs. post-menopausal subjects. The mean T_0 [TNF-α] for pre-menopausal subjects was not significantly different compared to post-menopausal subjects (P = 0.14). The mean T_1 [TNF-α] for pre-menopausal subjects was not significantly different compared to post-menopausal subjects (P = 0.66). The difference in mean cytokine response (e.g., ΔT_1-T_0) for [TNF-α] in pre-menopausal subjects compared to post-menopausal subjects was not statistically significant (P = 0.91).

DISCUSSION

In both pre-menopausal and post-menopausal participants, our data showed a statistically significant increase from T_0 and T_1 following orthodontic activation for all cytokines investigated. These results are in agreement with previous studies measuring cytokine response to orthodontic activation.[13,24,25] Since all participants

responded to orthodontic activation in a manner consistent with previous studies (increase in GCF inflammatory cytokine concentration), we are confident in the analytic comparison between pre- and post-menopausal groups.

When comparing cytokine response to orthodontic activation for pre- vs. post-menopausal groups, there were significant differences in mean [IL-1β]. There was a significant difference in [IL-1β] for pre-menopausal participants compared to post-menopausal participants at T_1 (12.66± 6.34 pg/mL vs. 23.38± 17.53 pg/mL, P< 0.039). However, there was not a significant difference for T_0 pre-menopausal [IL-1β] compared to T_0 post-menopausal [IL-1β] (7.99± 7.86 pg/mL vs. 10.56± 7.69 pg/mL, P= 0.26). It's been shown that *serum* IL-1β concentration is higher in healthy, post-menopausal females compared to healthy pre-menopausal females.[10] However, based on the results of this study, it does not appear IL-1β filters from the serum into the GCF in this sampling. Increased IL-1β is associated with periodontal inflammation, in the literature;[26] the participants of this study were periodontally healthy individuals. But, when an inflammatory insult is applied to the local periodontal environment, such as orthodontic activation, post-menopausal females show significantly increased levels of IL-1β compared to pre-menopausal females. This finding is reinforced by ΔT_1-T_0 data from the current study; there was a significant difference in GCF [IL-1β] response in pre-menopausal participants compared to post-menopausal for ΔT_1-T_0 (5.87± 4.13 vs. 14.86± 14.69, P= 0.05). Thus, orthodontic activation provides an inflammatory stress to the healthy periodontium, and post-menopausal subjects show a significantly increased inflammatory response for IL-1β

compared to the pre-menopausal subject response. This is finding is echoed by the results when Reinhardt et al.,[26] who found increased GCF IL-1β in post-menopausal females compared to pre-menopausal females in conditions active periodontal inflammation.

When comparing cytokine response to orthodontic activation for pre- vs. post-menopausal groups, there were no significant differences in [IL-6]. Specifically, there was not a significant difference for T_0 pre-menopausal [IL-6] compared to T_0 post-menopausal [IL-6] (1.88± 1.18 pg/mL vs. 1.74± 0.92 pg/mL, P= 0.64). Also, there was not a significant difference for T_1 pre-menopausal [IL-6] compared to T_1 post-menopausal [IL-6] (3.39± 1.33 pg/mL vs. 3.22± 1.64 pg/mL, P= 0.98), nor for ΔT_1-T_0 pre-menopausal [IL-6] compared to ΔT_1-T_0 post-menopausal [IL-6] (1.46± 1.15 pg/mL vs. 1.72± 1.64 pg/mL, P= 0.60).

When comparing cytokine response to orthodontic activation for pre- vs. post-menopausal groups, there were no significant differences in [TNF-α]. Specifically, there was not a significant difference for T_0 pre-menopausal [TNF-α] compared to T_0 post-menopausal [TNF-α] (2.78± 1.51 pg/mL vs. 2.2± 0.64 pg/mL, P= 0.14). Also, there was not a significant difference for T_1 pre-menopausal [TNF-α] compared to T_1 post-menopausal [TNF-α] (5.37± 3.2 pg/mL vs. 4.5± 3.47 pg/mL, P= 0.66), nor for ΔT_1-T_0 pre-menopausal [TNF-α] compared to ΔT_1-T_0 post-menopausal [TNF-α] (2.39± 2.73 pg/mL vs. 2.6± 2.99 pg/mL, P= 0.91). These authors could not find other studies investigating the differences of GCF TNF-α in pre- vs. post-menopausal females. A recent review, Montserrat Boronat-Catalá et al.[27] examined the 15 studies

completed in the last 10 years, which analyzed the salivary and GCF markers of inflammation. They found only 2 studies that analyzed TNF-α as a means to measure inflammation; however, neither of those studies had study populations comparable to the current study. In addition, they concluded that, at this time, there is only sufficient evidence to suggest IL-1β in saliva and/or gingival crevicular fluid can be used as a marker of the degree of inflammation.[27] The lack of data concerning GCF [TNF-α] and its relationship to menopause highlights the need for further study.

Other theories behind only seeing an increase in [IL-1β] are varied. One theory is that IL-1β is a more important player early in the bone-remodeling cascade as compared to IL-6 and TNF-α. At 24-hours post orthodontic activation, Uematsu et al.[13] found a mean of 0.88 pg/μg concentration for IL-1β, but for IL-6, the mean concentration was only 0.063 pg/μg at 24-hours post orthodontic activation. Even though both cytokines had increased significantly post activation, the amount of cytokine in the GCF clearly favored IL-1β. This pattern was echoed in our study in both the pre- and post-menopausal samples (Table IV: pre-menopausal T_1 mean IL-6 was 3.39 pg/mL compared to pre-menopausal T_1 mean IL-1β was 12.66 pg/mL; post-menopausal T_1 mean IL-6 was 3.22 pg/mL; post-menopausal T_1 mean IL-1β was 23.38 pg/mL). Of note, the units of measure are not identical in the two studies, but the relative difference between the two cytokines is similar in both the Uematsu study[13] (1996) and the study in question. However, our results disagree with the Uematsu study[10] for amounts of TNF-α relative to IL-1β. The Uematsu[13] data showed 0.88 pg/μg mean concentration for IL-1β at 24 hours post activation and 1.13

pg/μg mean concentration for TNF-α at 24-hours post activation. Conversely, in the current study, the concentration of TNF-α was much smaller relative to the concentration of IL-1β (pre-menopausal T_1 mean TNF-α was 5.37 pg/mL compared to pre-menopausal T_1 mean IL-1β was 12.66 pg/mL; post-menopausal T_1 mean TNF-α was 4.50 pg/mL; post-menopausal T_1 mean IL-1β was 23.38 pg/mL)

Another reason in the support of the increased IL-1β compared to IL-6 and TNF-α theory is IL-1β's high frequency of interaction with other cytokines.[28] During in vivo experiments, it's been shown that TNF-α, by itself, is less effective at inducing osteoclastogenesis. However, when coupled with IL-1β, TNF-α can synergize with RANKL to directly potentiate bone resorption by osteoclasts.[28] Similarly, osteoclasts induced by TNF–α formed initial resorption pits only in the presence of IL-1β.[29] Thus, it is likely that a higher concentration of IL-1β was found not only due to its own contribution toward remodeling, but also due to other members of the resorption cascade needing the assistance of IL-1β. Also, IL-1β, IL-6, and TNF-α have long been known to be part of the complement of resorptive cytokines; however, Pacifici et al.[10] suggested that IL-1β is a pivotal player in post-menopausal bone loss, since (i) IL-1β has an inverse relationship with bone mineral density, (ii) its production mirrors the rate of bone turnover in healthy and osteoporotic patients, and (iii) it's found at higher concentrations in untreated post-menopausal women than in untreated pre-menopausal women or estrogen/progesterone-treated post-menopausal women. Furthermore, Jimi et al.[30] found that IL-1β promotes the survival of osteoclasts through the activation of NF-

κB, which would mean as the resorptive process is starting, increased IL-1β would be produced to assist with not only osteoclast activation, but also survival.

There were also weaknesses due to subjects' self-reporting of health history. Potential underlying chronic conditions, which may modulate systemic inflammation, were not verified through lab tests nor correspondence with the subjects' physician(s). Additionally, patients were not screened for BMI or nutrition status – both of which can affect the body's systemic inflammation levels.[28,31] Another weakness with our data involves laboratory errors and compensations. Due to a labeling communication difficulty with the lab, it was incumbent upon the investigators to sort out the mislabeled vials as best as possible. The investigators corrected the lab's mislabeled samples to what they believed to be the appropriate samples; recovered samples were used when applicable toward means or standard deviations. However, those subjects who did not have *both* T_0 and T_1 samples were not included in the ΔT_1-T_0 calculations, which decreased the sample size available for analysis. After accounting for these laboratory complications, the following participants were available for statistical analyses: 14 pre-menopausal and 12 post-menopausal subjects contributed toward the mean and standard deviation calculations; 12 pre-menopausal and 11 post-menopausal subjects contributed toward t-test comparisons between the two groups.

Future studies are needed to further investigate the relationship between inflammatory cytokines and post-menopausal bone remodeling. GCF sample collection could be completed at various days after activation to discover if one

cytokine (IL-1β, IL-6, TNF-α e.g.) is more prominent than others at early activation vs. two-days or three-days post activation. This data also implies a correlation between orthodontic activation and biomarker change; however, since there was no measure of the amount of activation (or force) and only two time points to compare, there was no attempt to correlate the two variables. Future studies are needed to determine if increasing force leads to increased (or decreased) amounts of GCF IL-1β. Alternatively, cytokine concentration could be measured at multiple time points (1 hour post-activation, 6 hours post-activation, 12 hours post-activation, etc.) leading up to 24 hours to determine if a correlation exists. Also, GCF samples could be collected concurrently with serum samples to investigate any similarities or differences in systemic vs. local testing. Also, similar research could examine the effect of nutritional status on post-menopausal remodeling.

Conclusions

1) The GCF concentration of cytokines IL-1β, IL-6, and TNF-α increase after orthodontic activation in pre- and post-menopausal females.
2) Post-menopausal females with a healthy periodontium show a significantly increased inflammatory response for IL-1β compared to the pre-menopausal subject females.
3) There is not a statistically significant difference in GCF IL-6 and TNF-α cytokine response in pre- and post- menopausal women receiving orthodontic treatment.
4) It is worthwhile to invest in future studies investigating the effect of

menopause on inflammatory cytokines and orthodontic therapy.

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Fig 1. Graphical representation of means and standard deviations for [IL-1β], categorized by orthodontic activation time point. Within each time point is a visual comparison of pre-menopausal and post-menopausal inflammatory cytokine concentrations.

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Figure 1. Means and Standard Deviations, IL-1β

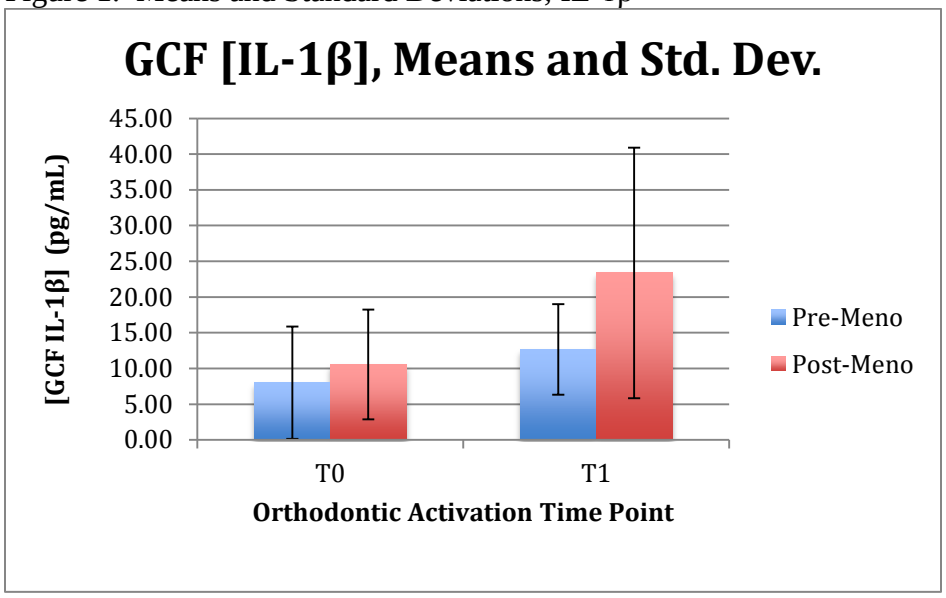


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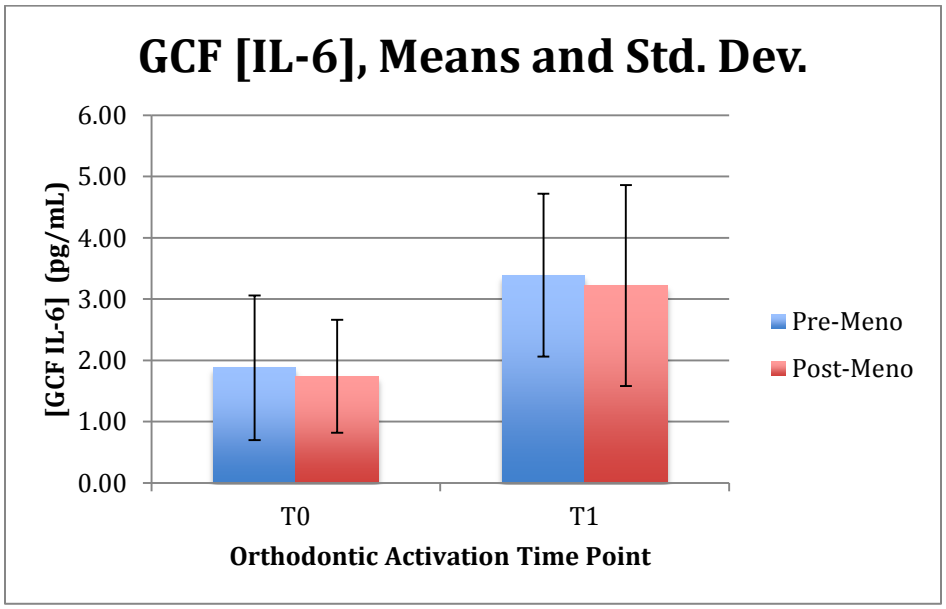


Figure 3. Means and Standard Deviations, TNF-α

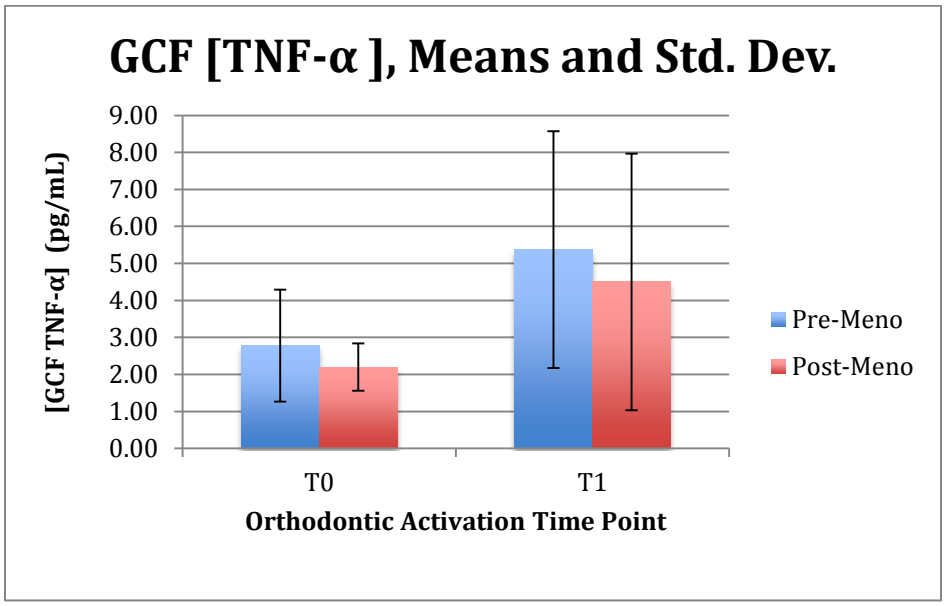


Table I.--Inclusion Criteria

Inclusion Criteria
≥21 years old
No Hormone replacement therapy
Bleeding On Probing ≤10% of sites
Plaque score ≤30%
≥2 months into orthodontic treatment
No patient report of systemic inflammatory condition (rheumatoid arthritis, Sjogren's syndrome, Crohn's disease, etc.)
No anti-inflammatory drug (NSAIDS, corticosteroids) in past 30 days

Table II.--Subject Demographics

Group	Number of subjects	Avg. Age	Age Range	Ethnicity
Pre-Menopausal	16	32	23-47	15 White 1 Asian
Post-Menopausal	14	63	58-80	13 White 1 Hispanic

Table III.--Coefficients of Determination (R^2)

Cytokine	R^2 Value
IL-1β	0.97
IL-6	0.98
TNF-α	0.96

Table IV.--GCF IL-1β, Measurements and Tests

Pre-Menopausal Subjects	IL-1β, T_0 (pg/mL)	IL-1β, T_1 (pg/mL)	IL-1β, ΔT_1-T_0 (pg/mL)
Mean	7.99	12.66	5.87
Std. Deviation	7.86	6.34	4.13
T-Test: To vs. T1, pre-meno		0.0005**	
Post-Menopausal Subjects	IL-1β, T_0 (pg/mL)	IL-1β, T_1 (pg/mL)	IL-1β, ΔT_1-T_0 (pg/mL)
Mean	10.56	23.38	14.86
Std. Deviation	7.69	17.53	14.69
T-Test: To vs. T1, post-meno		0.0082**	

**Denotes statistically significant

Table V.-- GCF IL-6, Measurements and Tests

Pre-Menopausal Subjects	IL-6, T_0 (pg/mL)	IL-6, T_1 (pg/mL)	IL-6, ΔT_1-T_0 (pg/mL)
Mean	1.88	3.39	1.46
Std. Deviation	1.18	1.33	1.15
T-Test: To vs. T1, pre-meno		0.0002**	
Post-Menopausal Subjects	IL-6, T_0 (pg/mL)	IL-6, T_1 (pg/mL)	IL-6, ΔT_1-T_0 (pg/mL)
Mean	1.74	3.22	1.72
Std. Deviation	0.92	1.64	1.37
T-Test: To vs. T1, post-meno		0.0020**	

**Denotes statistically significant

Table VI.-- GCF TNF-α, Measurements and Tests

Pre-Menopausal Subjects	TNF-α, T_0 (pg/mL)	TNF-α, T_1 (pg/mL)	TNF-α, ΔT_1-T_o (pg/mL)
Mean	2.78	5.37	2.39
Std. Deviation	1.51	3.2	2.73
T-Test: To vs. T1, pre-meno		0.0047**	
Post-Menopausal Subjects	TNF-α, T_0 (pg/mL)	TNF-α, T_1 (pg/mL)	TNF-α, ΔT_1-T_o (pg/mL)
Mean	2.2	4.5	2.6
Std. Deviation	0.64	3.47	2.99
T-Test: To vs. T1, post-meno		0.0118**	

**Denotes statistically significant

Table VII.--T-Tests: Pre- vs. Post-Menopausal Groups

Pre- vs. Post-menopausal T-Test Variables	P-Value
IL-1β Pre- vs. Post-meno, T_0	0.26
IL-1β Pre- vs. Post-meno, T_1	0.0388**
IL-1β Pre- vs. Post-meno, ΔT_1-T_0	0.0544**
IL-6 Pre- vs. Post-meno T_0	0.64
IL-6 Pre- vs. Post-meno T_1	0.98
IL-6 Pre- vs. Post-meno, ΔT_1-T_0	0.60
TNF-α Pre- vs. Post-meno T_0	0.14
TNF-α Pre- vs. Post-meno T_1	0.66
TNF-α Pre- vs. Post-meno, ΔT_1-T_0	0.91

**Denotes statistically significant