Trauma and Cytokines: Gingival Crevicular Fluid Biomarkers in Traumatized Permanent Incisors - A Pilot Investigation

THESIS

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By

Christopher G Rosenvall

Graduate Program in Dentistry

The Ohio State University

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Master's Examination Committee:

Dennis McTigue, Advisor

Purnima Kumar

Ashok Kumar

Kumar Subramanian
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Abstract

Extensive research has yielded unpredictable long-term results for traumatized teeth. While epidemiological research in the area of treatment of traumatized teeth is well documented, descriptive studies focusing on biological predictors of healing are non-existent. The objective of this study is to determine if any variation exists between normal healing and pathology in traumatized teeth in growing children that could lead to early predictors of pathology. This could ultimately help to decrease cost and time for patients and their families.

In order to determine a baseline of biomarkers of traumatized teeth, we retrospectively examined cytokine levels in gingival crevicular fluid (GCF) following trauma of permanent mature anterior teeth in growing children. GCF was collected from each injured tooth and a non-injured control tooth in the same arch. Injured teeth were categorized as displaced or non-displaced injuries. A total of 48 injured teeth from 34 patients were examined. 27 cytokines were analyzed, and levels of cytokines were found to be significantly different in injured teeth compared to control teeth. Additionally, levels of specific cytokines were significantly higher in displaced teeth compared to non-displaced injuries. The results of this study lay a foundation for further studies that can provide information for a possible early test to predict healing of traumatized teeth.
Dedication

This document is dedicated to my family.
Acknowledgments

My sincere appreciation for the many hours of direction and guidance given by Dr. Dennis McTigue, Dr. Purnima Kumar, Dr. Kumar Subramanian, and Dr. Ashok Kumar.
Vita

2007.......................................................B.A. English, Brigham Young University

2011.......................................................D.D.S., Baylor College of Dentistry

2011 - Present .....................................Pediatric Dental Resident, Graduate

                                          Teaching Associate, Department of
                                          Dentistry, The Ohio State University

Fields of Study

Major Field: Dentistry
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Chapter 1: Introduction

Trauma to the permanent anterior teeth is extremely common worldwide. It is estimated that trauma composes 5% of all injuries for which people seek treatment, with at least 25-35% all school-aged children suffering from some type of trauma to the teeth. The majority of dental injuries in young children and adolescents occur to the anterior teeth. Between the ages of one to two, the main cause of dental trauma is falling while learning to walk. As a child gets older, the leading sources are from falls, sports, fights, and traffic accidents. When immature teeth are injured at a young age, the goal is to save the teeth and prevent or minimize further damage to the teeth and supporting structures. Delaying treatment or improper treatment can cause a lasting impact on the physical, emotional, and psychological health of the child.

The International Association of Dental Traumatology (IADT) publishes guidelines detailing the currently accepted methods for diagnosis and treatment of injuries to the dentition. Dental trauma can be broadly categorized as injuries to the actual teeth – such as crown fractures – and luxation injuries, which damage the periodontal support of the teeth. A brief description of each injury and treatment recommendations based on the IADT guidelines are as follows.

**Crown Fractures:** Fractures are very common, comprising 26-76% of all dental injuries, although some argue that the actual prevalence is much higher because most fractures are not treated due to economic reasons or lack of access to care.
fracture only involves the enamel (Class I fracture) the prognosis is very favorable, with recommended treatment being to smooth off the rough edges of enamel or restoring the lost tissue with a restoration. If the fracture also includes dentin (Class II fracture), it is recommended to seal over the exposed area to prevent bacterial contamination of the dentinal tubules. An exposure of the pulp to the oral cavity (Class III fracture), provides direct access for bacterial invasion into the pulp. In young patients with immature teeth, it is advantageous to preserve pulp vitality by pulp capping or a partial pulpotomy so that the teeth can continue to mature. In mature teeth, root canal treatment is usually the preferred treatment. A crown fracture may also be accompanied by a luxation injury.

**Luxation injuries:** Luxation injuries, or trauma to the periodontium supporting the teeth, are more common in the primary dentition, although they still occur frequently in the permanent teeth\(^1\).\(^6\). Injuries to the periodontium can range from a mild concussive injury to one that displaces the tooth in the socket and fractures the alveolus. Concussion and subluxation injuries do not produce tooth displacement in the bony socket – concussion is an injury to the structures supporting the tooth without causing mobility, while subluxation is a more serious injury that causes some loosening of the tooth in the alveolus. Extrusive luxation causes a tooth to partially extrude out of the socket in an axial direction, while the periodontal ligament fibers (PDL) and neurovascular supply to the tooth are stretched or ruptured. A lateral luxation is a displacement in the tooth in the socket, usually accompanied by an alveolar fracture. An intrusive luxation causes apical dislocation of the tooth in the socket, which crushes the PDL and neurovascular supply. The IADT recommends repositioning luxated teeth, stabilizing them with a flexible splint.
for about two weeks, and regular trauma follow-ups\textsuperscript{1}. Severe luxation injuries are often associated with an alveolar fracture and complications such as root resorption, ankylosis, and necrosis of the pulp\textsuperscript{1, 7, 8}. Finally, an avulsion is when the tooth is completely displaced from the socket. The PDL and neurovascular supply are totally severed, and major complications are common even if the tooth is reimplanted\textsuperscript{9}. Avulsion injuries require reimplantation as soon as possible to preserve vital PDL cells. If the extra-oral dry time is over one hour, it is recommended to scrape the dry PDL cells off the root and reimplant. The longer an avulsed tooth is out of the mouth, the more likely there will be long-term consequences to the tooth\textsuperscript{1}.

After a dental injury a person is at risk for complications, depending on the extent of the injury and the stage of root development. A simple, uncomplicated crown fracture has an excellent prognosis. A luxation injury, however, can cause lasting damage due to destruction to the PDL and vascular supply of the tooth. A luxated immature tooth with an open root apex has a greater possibility that its neurovascular supply can revascularize or withstand some displacement and remain vital\textsuperscript{8, 10}. However, if the tooth is mature with a closed apex, the supply is more likely to be permanently damaged and develop pulpal necrosis. A necrotic pulp often becomes infected with bacteria. Bacterial invasion further complicates the healing process and can initiate an inflammatory response that leads to bone and root resorption and loss of the tooth. Extensive damage to the PDL creates “welding” of the tooth to the bony alveolus, a process called ankylosis. If a large area of the root becomes ankylosed, it can be replaced with bone and the tooth is lost.
In crown fractures that do not expose the pulp, inflammatory changes are temporary as long as the vascular supply is intact and the pulp is able to defend against a bacterial insult\textsuperscript{11}. Pulpal necrosis is rare in Class I and Class II fractures unless there is also a periodontal injury that damages the blood supply to the tooth, since a compromised blood supply makes it difficult for the vital tissue to defend against bacteria. A periodontal injury associated with a simple fracture increases the chance of developing pulpal necrosis from 0\% to 28\%\textsuperscript{5}. This may pose a diagnostic challenge to the clinician, because it can be easy to overlook a periodontal injury and focus more on restoring the obvious fracture\textsuperscript{8}.

A luxation injury greatly increases the risk of developing pulpal necrosis – in some injuries it nears 100\% \textsuperscript{8}. A necrotic pulp is susceptible to bacterial infection, which may lead to constant inflammation that promotes root resorption, tissue destruction and loss of the tooth. An injured, necrotic tooth infected with bacteria needs immediate endodontic therapy to remove the source of infection and inflammation. The following chart illustrates the risk of developing necrosis after dental injuries \textsuperscript{8,12}: 
<table>
<thead>
<tr>
<th>Injury</th>
<th>Pulp necrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concussion</td>
<td></td>
</tr>
<tr>
<td>Open apex</td>
<td>0</td>
</tr>
<tr>
<td>Closed apex</td>
<td>4</td>
</tr>
<tr>
<td>Subluxation</td>
<td></td>
</tr>
<tr>
<td>Open apex</td>
<td>0</td>
</tr>
<tr>
<td>Closed apex</td>
<td>15</td>
</tr>
<tr>
<td>Extrusion</td>
<td></td>
</tr>
<tr>
<td>Open apex</td>
<td>9</td>
</tr>
<tr>
<td>Closed apex</td>
<td>55</td>
</tr>
<tr>
<td>Lateral luxation</td>
<td></td>
</tr>
<tr>
<td>Open apex</td>
<td>9</td>
</tr>
<tr>
<td>Closed apex</td>
<td>77</td>
</tr>
<tr>
<td>Intrusion</td>
<td></td>
</tr>
<tr>
<td>Open apex</td>
<td>63</td>
</tr>
<tr>
<td>Closed apex</td>
<td>100</td>
</tr>
<tr>
<td>Avulsion</td>
<td></td>
</tr>
<tr>
<td>Open apex</td>
<td>70</td>
</tr>
<tr>
<td>Closed apex</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1. Prevalence of pulpal necrosis based on root development and trauma

It is extremely important that immature teeth are treated appropriately in a timely manner after a traumatic injury. A necrotic tooth with immature root development and a large, open apex is very challenging to treat endodontically. First, the necrotic pulp tissue cannot be removed using standard root canal protocol with endodontic files and aggressive cleaning because the root walls are too thin. Second, after removing the infected tissue it is difficult to fill the root canal with an open apex because there is no stop to hold in the filling material. Third, even if the endodontic procedure is successful, the roots are thin, weak and have a high risk of fracture in the future. Thus, the dental professional often faces a difficult dilemma when treating traumatized immature teeth. The dentist will want to avoid endodontic therapy if the immature tooth is vital,
since a vital tooth will continue root development, strength and thickness. It is estimated that up to 80% of teeth that are treated endodontically before full root development are later lost to crown and root fractures. However, if an immature tooth becomes necrotic, root resorption can be swift, aggressive and lead to the loss of the tooth. How does one confidently determine that the tooth is necrotic and needs endodontic treatment? Unfortunately, the answer is not as clear as we would like.

Currently, a dental practitioner must rely on sometimes murky clinical and radiographic signs and symptoms to determine if an immature tooth is necrotic. Signs such as arrested tooth development, discoloration, external inflammatory root resorption, pain to percussion, and absence of response to cold stimulation are all used to make a judgment if the tooth is necrotic. However, FM Andreasen argues that tooth luxations and crown fractures are the most over treated trauma entities encountered in dentistry today. As the tooth heals from trauma, it may show signs and symptoms of pulp necrosis such as loss of pulpal sensibility, discoloration and radiographic resorption of the root – but the pulp is still vital. After injury there is a race, or tug-of-war, between pulpal healing and necrosis, and it can be difficult to determine which process is dominating. Often the processes of healing and necrosis can be happening simultaneously. FM Andreasen asserts that the risk of pulp necrosis should be determined by the stage of root development and the extent of the injury, and not solely by clinical variables because nearly all conventional signs could be indications of either pulp healing or necrosis. The dental professional is often limited to making the best educated guess for the course of treatment. Clearly, a better ability to diagnose, monitor
and predict healing in dental trauma, especially to immature teeth, would be immensely beneficial.

A possible new diagnostic tool may be to analyze host biomarkers in the gingival crevicular fluid of a traumatized tooth. Gingival crevicular fluid (GCF) is an inflammatory exudate, chiefly composed of serum, which can be collected in the gingival sulcus. As the fluid passes through the gingival tissue, it picks up host-derived enzymes, inflammatory mediators and tissue breakdown products which can be analyzed and examined by collecting it in the gingival crevice\textsuperscript{16-18}. Of particular interest is the presence of inflammatory mediators and cytokines in the GCF. An exciting area of research has delved into the analysis of cytokines produced throughout the human body. Cytokines play a central role in regulating inflammation and the healing process – and also as mediators of pathology. Researchers have examined the function of cytokines in everything from heart disease to diabetes to burn wounds. There is a potential that examining cytokines in GCF samples taken from traumatized teeth could illuminate the healing process of the tooth and assist dentists in treatment decisions. A basic understanding of the healing process and the role of cytokines is important.

There are three classic stages of wound healing: inflammation, proliferation, and remodeling – although in reality the three phases are more of a continuum than discrete steps. After an injury, local tissues release factors that initiate the intrinsic and extrinsic coagulation pathways. A blood clot is formed, and platelets adhere to the clot and begin releasing mediators of wound healing. These mediators, or cytokines, attract leukocytes and initiate the inflammatory response\textsuperscript{19,20}.
The first cells to arrive to an injured site are neutrophils $^{20,21}$. They are attracted to the area by chemotactic proteins, or cytokines, released from platelets and other local cells in the wound area. Their major role is to sweep the area clean of bacteria to protect against infection. Neutrophils play a critical role in inflammation and wound healing, but they do expel products that cause tissue destruction and are linked to chronic inflammation. They also release inflammatory mediators and cytokines that attract other cells, especially monocytes/macrophages$^{19}$.

Monocytes are seen after initial trauma and arrive after the neutrophils. These cells are formed in the bone marrow and are drawn to wounds via chemoattractants. Injured tissues, platelets, neutrophils, mast cells and lymphocytes all release chemicals that attract monocytes. As the monocytes enter the wound area, they are activated into “inflammatory macrophages” that destroy and phagocytize bacteria and necrotic tissue$^{22}$. They release many cytokines that regulate inflammation and attract other cells essential to healing $^{19,20}$. Lymphocytes, or T and B cells, also arrive and produce cytokines that assist in angiogenesis and healing, as well as attract fibroblasts to the site. Macrophages and lymphocytes, through the release of cytokines, begin the process of proliferation and remodeling stages of wound healing.

In the proliferation and remodeling stages of wound healing, fibroblasts proliferate and produce connective tissue to repair and restore function to the injured area. Growth factors and cytokines guide fibroblasts and endothelial cells as they replace granulation tissue and begin angiogenesis. As the wound heals, cytokines continue to be essential in restoring function of cells and injured tissues. The success of wound healing
depends on the complex relationship of cytokines interacting together to regulate cellular functions\textsuperscript{19, 23}.

Trauma to the teeth and supporting structures initially causes an inflammatory response. In the case of a tooth fracture without a periodontal injury, the pulp responds by laying down reparative dentin as a barrier and protection from bacterial insult \textsuperscript{5}. Pulpal inflammation actually increases the outward flow of dentinal fluid and prevents bacteria from invading the pulp \textsuperscript{5, 11}. Trauma that exposes the pulp to the oral cavity increases the risk of infection. If the vital tissue is able to resist bacterial infection, layers of fibroblasts form over the exposed area and start to calcify. Within one month, a dentinal bridge is formed that will prevent open exposure of the pulp to the mouth. Studies have shown, though, that irrespective of pulp exposure, pulp survival is still entirely dependent on the presence of an associated luxation injury \textsuperscript{5, 10, 11}. A luxation injury may damage the pulp’s blood supply and its ability to defend against bacterial invasion is dramatically weakened.

If the supporting structures of the teeth are injured in a luxation injury, the damaged PDL is removed along with bone and cementum. The damaged tissue will be restored with new cementum, PDL and bone if the injury is not severe. However, significant loss of cementoblasts, PDL, and epithelial rests of Mallasez results in a large denuded area on the root\textsuperscript{15}. The bare area is chemotactic to hard tissue resorbing cells which can lead to root resorption. This process may be temporary, as the activated macrophages and osteoclasts initially remove the damaged tissue and then stop, and normal healing takes place\textsuperscript{10}. If the area damaged on the root is minor, new cementum
and PDL will cover the area over time. A denuded area of the root alone is not enough to sustain root resorption for more than 2-3 weeks. However, constant inflammatory stimulation, such as products from bacterial infection, will stimulate continual resorption of root and bone.

If the pulp becomes necrotic and infected with bacteria, the tooth is at risk for aggressive inflammatory root resorption. Toxins from bacteria in the pulp can leak through the dentinal tubules to cause continued inflammation and hard tissue resorption by osteoclasts. If the bacterial stimulation is removed through endodontic therapy, the process stops. The resulting defect is then repaired by cementum or bone, depending on the type of tissue found next to the resorption site. If the bacterial stimulation is not removed, the activation of osteoclasts and resorption continues, and the tooth can ultimately be lost.

Significant damage to the periodontium creates a large denuded area on the root. Bone-producing cells are attracted to the area and directly attach to the root surface before cementum has a chance to form – this can form a “welding” of the tooth to bone, or ankylosis. When less than 20% of the root surface is bare, a reversible ankylosis occurs. But in larger injuries, permanent ankylosis is likely. The ankylosed tooth becomes part of the bone remodeling system and is slowly replaced with bone, a process called replacement resorption. This is often seen in avulsion injuries, when most of the PDL cells are destroyed or desiccated by injury and time spent out of the mouth.

Cytokines released by local cells were initially investigated for their role in inflammation, but it is becoming clear that they are critical throughout the entire healing
Cytokines are small proteins secreted from many cell types that regulate the nature, intensity, and duration of the immune response. While many cells secrete these biomarkers, the predominant producers are helper T cells (T<sub>H</sub>) and macrophages. These proteins are involved in virtually every aspect of immunity and inflammation and are active in all stages of wound healing. There are many known cytokines, all of which create a vast, complex and interconnected network. A single cytokine can have different effects on different cells, synergize with another, act as an antagonist to another cytokine, or stimulate the production of other cytokines – the function of each cytokine in relation to each other is still being investigated. A table of significant cytokines can be found in the appendix.

Cytokines can be roughly divided into “pro-inflammatory” and “anti-inflammatory” groups based on their general function. Pro-inflammatory cytokines are principally produced by activated macrophages. For example, IL-1β, IL-6, and tumor necrosis factor (TNF)-α are considered pro-inflammatory mediators. Conversely, IL-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13 can suppress the production of inflammatory cytokines. There is a “balance” between the two groups of cytokines that ultimately determines the outcome of healing and disease. During initial inflammation, the pro-inflammatory cytokines are crucial for the healing process. Over time, these cytokines should be reduced and regulated by anti-inflammatory cytokines. However, changes in cytokine expression or function may lead to continual, prolonged inflammation. This creates chronic inflammation and pathology. It is important to note that when describing cytokines, one should not firmly divide them into the two
groups “pro” or “anti” inflammatory. Some cytokines are pleiotropic in nature. For example, transforming growth factor (TGF)-β and IL-6 are either anti-inflammatory or pro-inflammatory depending on the situation. TNF-α and IL-1β promote inflammation but also help the body fight against infection, which is vital for healing.

Some cytokines are known to induce chemotaxis and are called chemokines. Chemokines are small proteins under the cytokine family that direct the movement of circulating cells to sites of inflammation and injury. There are over 52 different chemokines identified. They are crucial in stimulating the adaptive immune response and contributing to a variety of diseases. Human chemokines are divided into four families on the basis of their structure and function, with the two larger groups being the CC and CXC chemokines. The largest family, the CC chemokines, is so designated because the first two of four cysteine residues are adjacent to each other. CC chemokines attract mononuclear cells to sites of chronic inflammation. Well-studied CC chemokines include monocyte chemoattractant protein 1 (MCP-1, or CCL2) that attracts and activates monocytes, dendritic cells, memory T cells, and basophils. Other CC chemokines include macrophage inflammatory protein (MIP) 1-α (CCL3), MIP-1β (CCL4), and RANTES (CCL5).

Another significant group is the CXC chemokines, which have a single amino acid residue between the first two cysteines. Interleukin-8 (CXCL8) is a well-studied CXC chemokine. They chiefly attract polymorphonuclear leukocytes to sites of acute inflammation, and activate and recruit monocytes to injured areas. A table of a few important chemokines is listed below.
Research of cytokines throughout the human body illuminates their many functions. In the past, studies have focused on their participation in initial inflammation, but they have recently been examined for their roles in other biological processes. In addition to inflammation, cytokines and chemokines are now recognized as important regulators in immune homeostasis. They are also associated with embryonic development, angiogenesis and cutaneous wound healing. For example, it was found that levels of IL-1α, IL-6, TNF-α, MIP-1α and PDGFα were higher in murine models with cutaneous injuries, and that human patients with sepsis after burns had higher levels of IL-10. Synovial fluid in inflamed human knees due to trauma and inflammatory disease had high levels of RANTES and MCP-1. These cytokines attract mononuclear phagocytes and leukocytes to the inflamed area, and are regulated by other cytokines.
such as IL-1 and TNF-α\textsuperscript{39}. They are involved in every step of healing, from initial inflammation to regeneration and repair of tissues. Cytokines are recognized to have many functions in the healing process and homeostasis, not just in acute inflammation.

Cytokines and chemokines are also involved in pathological processes. Initially, inflammatory cytokines are released to combat infection, and inflammation is an essential step in the healing process. But continual infection or insult leads to the dysregulation of the immune system and the balance between inflammation and anti-inflammation becomes tilted toward chronic, damaging inflammation\textsuperscript{40}. The anti-inflammatory mediators do not provide sufficient control over pro-inflammatory conditions, or overcompensate and smother the immune response which exposes the host to systemic infection – and pathology results\textsuperscript{32,41}. Studies suggest that this balance of pro-inflammatory and anti-inflammatory cytokines may be genetically determined\textsuperscript{32}.

Cytokines and chemokines have been associated with a variety of diseases that have prominent inflammatory components, such as rheumatoid arthritis, asthma, psoriasis and skin inflammation\textsuperscript{33,42}. Recent studies have also linked chemokines to multiple sclerosis, atherosclerosis, Type II diabetes and cancer\textsuperscript{33}. Chronic inflammation can actually lead to tumor formation, and many cancers up-regulate expression of chemokines as a way to attract nutrients, supporting cells, blood supply, and to metastasize\textsuperscript{28,43,44}. Adipose cells also secrete cytokines which may cause insulin resistance and increase the risk for Type II diabetes\textsuperscript{33,43,45,46}. Again, there is some evidence that one’s genetics may play a role in the type and amount of cytokines expressed. For example, cytokine polymorphisms have been found in those with Type II
diabetes and may be a way to identify individuals who may develop the disease in the future.

Of interest for dental trauma treatment is the role of cytokines in bone healing and pathology. Following injury to the bone tissue, high levels of pro-inflammatory signals are released: IL-1, IL-6, IL-11, IL-18, TNF-α and others. These cytokines attract inflammatory cells, promote angiogenesis, stimulate osteoprogenitor cells to release bone morphogenetic proteins, and help mesenchymal stem cells proliferate and differentiate. Over time, inflammation should subside and TGF-β2, -β3, bone morphogenetic proteins (BMPs) and others induce cartilage and bone formation. Osteoprogenitor cells differentiate into osteoblasts that express IL-1, IL-6, IL-11 and other cytokines that stimulate osteoclast formation. Osteoblasts and osteoclasts work together to remodel and replace injured bone.

Osteoclasts appear to be hematopoietic cells that arise from the monocyte-macrophage lineage. Osteoclasts are principally regulated by osteoblasts, and can resorb bone, cartilage, and dentin. They are recruited by cytokines and chemokines MCP-1(CCL2), MIP-1α(CCL3), and SDF-1(CXCL12) to the injured area. Additionally, IL-1 and TNF stimulate the inflammatory process which leads to osteoclast recruitment and bone resorption. MCP-1, MIP-1α, and SDF-1 also induce osteoclast differentiation, activate their resorption activity, and help with their survival. The list of cytokines that help in the development of osteoclasts is lengthy: IL-1, IL-3, IL-6, IL-11, TNF, granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor.
(LIF), and stem-cell factor (SCF) have all been implicated. The regulation of osteoclasts and bone resorption is obviously complex and interdependent.

Osteoclasts are principally regulated through the RANK/RANKL/OPG system. RANK ligand (RANKL) is a member of the tumor necrosis factor ligand family. Most factors that stimulate osteoclast formation and activity do so indirectly by up-regulating RANK ligand expression. The RANK ligand binds to the RANK receptor on osteoclast precursors, and osteoclasts are matured and activated. RANKL is produced by osteoblasts and stromal cells. Conversely, OPG is a ligand produced by osteoblasts that binds and neutralizes RANKL, which blocks osteoclastogenesis – it is termed a “decoy” receptor. Many cytokines interact with the RANK/RANKL/OPG system and influence the activity of osteoclasts and bone resorption. For example, IL-1, which is released during inflammation, fuels formation of RANKL and promotes bone resorption. BMP, TGF-β, and IFN-γ depress RANKL production, increase OPG production and stop bone resorption. Below are some of the cytokines that either enhance or depress osteoclast activity:
Cytokines that **enhance** osteoclast activity:\(^\footnote{49}\):

<table>
<thead>
<tr>
<th>Name</th>
<th>Produced by</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Macrophages, marrow stromal cells</td>
<td>Stimulates bone resorption, implicated in pathological conditions with bone loss</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Monocytes, endothelial cells, fibroblasts</td>
<td>Early osteoclast development – drives osteoclast precursor to osteoclast lineage (^\footnote{54})</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Macrophages</td>
<td>Stimulates osteoclast bone resorption</td>
</tr>
<tr>
<td>TNF (α and β)</td>
<td>Macrophages, lymphocytes</td>
<td>Potentiates IL-1 effect on osteoclast formation</td>
</tr>
<tr>
<td>IL-6</td>
<td>Monocytes, osteoclasts, osteoblasts</td>
<td>Induces osteoclast formation from osteoclast precursors, associated with Paget’s disease of the bone</td>
</tr>
<tr>
<td>IL-11</td>
<td>Marrow stromal cells</td>
<td>Induces RANKL expression on osteoblasts</td>
</tr>
<tr>
<td>RANTES</td>
<td>Macrophages, T-cells, platelets</td>
<td>Increases RANKL and osteoclast migration, but is also anabolic for osteoblasts</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophages, lymphocytes, fibroblasts</td>
<td>Increases RANKL and osteoclast migration, but is also anabolic for osteoblasts</td>
</tr>
</tbody>
</table>

Table 3. Cytokines that enhance osteoclasts

Cytokines that **inhibit** osteoclast activity:

<table>
<thead>
<tr>
<th>Name</th>
<th>Cell producing cytokine</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Osteoclast, osteoblast</td>
<td>Stimulates osteoblast bone formation, potent inhibitor of osteoclast bone resorption</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>T-cells, NK cells</td>
<td>Inhibitor of bone resorption by itself</td>
</tr>
<tr>
<td>IL-4</td>
<td>T-cells</td>
<td>Inhibits bone resorption</td>
</tr>
<tr>
<td>IL-18</td>
<td>T-cells</td>
<td>Induces IFN-γ</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoblasts</td>
<td>Decoy receptor for RANKL</td>
</tr>
<tr>
<td>BMP</td>
<td>Marrow stromal cells</td>
<td>Activates osteoblasts(^\footnote{55})</td>
</tr>
</tbody>
</table>

Table 4. Cytokines that inhibit osteoclasts
Cytokines are also implicated in bone pathology, which can illustrate their role in dental trauma. Bone destruction in patients with metastatic breast cancer is triggered by local production of cytokines secreted by tumor cells that stimulate osteoclast formation, such as IL-1β, IL-6, IL-8, and IL-11. Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation of synovial joints and progressive destruction of cartilage and bone. In those suffering from rheumatoid arthritis, high levels of TNFα and IL-1 initiate inflammation in the joints and recruit macrophage infiltration. Macrophages both stimulate and act as a source of precursors for osteoclast destruction. Secondary pro-inflammatory chemokines, proteases, and reactive ions interact to erode bone and cartilage. Higher levels of CC chemokines, such as MCP-1(CCL2), MIP-1α(CCL3), and RANTES(CCL5) have been found in joints of patients with rheumatoid arthritis. Inflammatory stimulated osteoblasts and other cell types release MIP-1α and RANTES that contribute to recruitment, RANKL development, and migration of osteoclasts to the joint and increases inflammatory bone loss. High levels of inflammatory chemokines stimulate osteoclasts via the RANK/RANKL system that eventually causes joint destruction.

Cytokines are also involved in osteoporosis. In normal bone remodeling, osteoclasts adhere to bone and remove it by acidification and proteolytic digestion. Osteoblasts then occupy the area and form new bone matrix. In healthy bone, this process is in harmony so that bone is remodeled but not lost. However, individuals suffering from osteoporosis have an imbalance between bone resorption and formation, and bone volume and strength is lost over time. Studies have implicated a higher level of
IL-6 and IL-11 in the progression of osteoporosis\textsuperscript{51}. It seems clear that cytokines are intimately involved in inflammatory bone loss throughout the body. They are also critical in bone loss due to inflammation in the oral cavity.

Cytokines have been intensely studied for their role in periodontal disease, endodontic lesions and root resorption due to orthodontic treatment. Periodontal disease is an inflammatory disease that causes destruction of bone and supporting tissues of teeth. The inflammation is triggered by the presence of bacteria, but bacteria alone are not enough to cause destructive periodontal disease. Not everyone is equally susceptible to the disease, which leads many to believe there are intrinsic differences in the host immune response\textsuperscript{42, 57, 58}. For example, one study found a two-fold surge in RANTES in untreated periodontal patients; the amount of RANTES did not decrease after therapy, which may indicate a genetic factor that increases the risk of periodontal disease\textsuperscript{58}. Researchers believe that analyzing host cytokines may help diagnose, treat, and predict the course of periodontitis better.

Continual inflammation from plaque and bacteria around the teeth tips the balance of the immune response to chronic inflammation and tissue destruction. Studies have found an increase of chemokines such as MIP-1\(\alpha\) (CCL3) in diseased periodontium\textsuperscript{59}. MIP-1\(\alpha\) attracts monocytes to the site of inflammation. The monocytes become activated “inflammatory macrophages” and interact with osteoblast-like cells such as PDL fibroblasts. The PDL fibroblasts can either support bone formation or bone destruction. Pro-inflammatory cytokines such as IL-1 and TNF-\(\alpha\) shift the phenotype of PDL fibroblasts to cells that favor bone resorption. Studies have shown that patients suffering
from periodontal disease have higher levels of IL-1, TNF-α, IFN-γ, and IL-6\textsuperscript{42, 60-62}. Macrophages and T\textsubscript{H}-1 cells are potent sources of these resorptive cytokines, which promote continual bone resorption\textsuperscript{59}. IL-1 and TNF-α are important because they stimulate the differentiation of macrophages into osteoclasts both directly and through the RANK ligand\textsuperscript{42}. Thus, activated macrophages interact with PDL fibroblasts and act like osteoclasts and destroy bone. An interesting study by Bloemen showed that just a single exposure of IL-1β can shift the phenotype of osteoblast-like cells from one that favors bone formation to one that supports bone destruction\textsuperscript{54}. Studies have shown that periodontal treatment reduces the levels of inflammatory cytokines, namely IL-1, IL-2, IL-3, IL-6, IL-7, IL-8, IL-12, MCP-1, MIP-1α, and INF-γ, which supports the theory that cytokines are important mediators of the disease\textsuperscript{40, 63}.

Cytokines are also involved with implant periodontitis. A popular restorative option for a lost tooth is to surgically place a dental implant, which is a titanium post that is embedded in the jaw and osseointegrates to the bone. However, patients with implants can also suffer from bacterial inflammation and bone destruction almost identical to periodontitis – this disease is termed implant periodontitis or “peri-implantitis.” This inflammatory reaction can cause the loss of supporting bone and lead to a failed implant\textsuperscript{64}. Studies have shown higher levels of IL-1β, IL-6, IL-8, IL-22, IL-23 and TNF-α in implants affected by peri-implantitis\textsuperscript{16, 64, 65}. Certain cytokines appear to actively propagate and support periodontal disease in natural teeth and in implants. In the future, we may be able to analyze these cytokines and diagnose periodontitis at an earlier stage or to predict the course of the disease.
Another possibility for using cytokines as a diagnostic tool is in orthodontic treatment. A common consequence of using orthodontic forces to move teeth is root resorption. This can be a minor occurrence, or it could be severe enough to cause questionable long-term prognosis of the affected teeth. The exact mechanism of sterile root resorption is not clearly understood, but there is evidence that inflammatory cytokines are involved. Orthodontic forces produce an inflammatory response. Osteoblasts and PDL fibroblasts under orthodontic mechanical stress release IL-1, IL-6, IL-8 and TNF-α. Other studies have shown that MCP-1, MIP-1α, M-CSF and RANTES are also involved. As described previously, all these cytokines are known to induce osteoclast formation and activity, thus stimulating root resorption. Zhang showed that IL-1 and TNF-α induces root resorption in rats, and that blocking these two cytokines reduced the resorption. Inflammatory cytokines are important factors in orthodontic root resorption, and monitoring them may help clinicians diagnose the resorption at an earlier stage.

As described earlier, there may be genetic polymorphisms that influence the type and amount of cytokines released after inflammation, and thus moderate the balance between healing and pathology. This may explain why some individuals seem to suffer from certain diseases while others do not. For example, cytokine polymorphisms were found in individuals with Type II diabetes. The literature about polymorphisms influencing periodontal disease is still in its infancy. Soedarsono looked at single nucleotide polymorphisms in RANKL and OPG in Japanese patients with periodontitis and found no significant associations with aggressive periodontitis. However, other
studies have found a link between polymorphisms on the IL-1 gene and chronic periodontitis$^{68-70}$. Although more research is needed, polymorphism or gene cluster variations may influence the risk of suffering from periodontal disease or the progression of tissue destruction because they impact the type or amount of inflammatory cytokines produced$^{42,69}$.

One of the limitations in identifying periodontal disease and orthodontic root resorption is that clinical measurements only provide information about the pathology’s effects that have already occurred$^{18,61}$. For example, a clinician will measure probing depth, clinical attachment levels, plaque index, bleeding on probing, and radiographic findings to identify periodontal disease, but these findings do nothing to diagnose the current stage of the disease or to predict its progression. Furthermore, a patient may present with clinical signs of periodontal destruction, but the disease has stopped$^{61}$. In other words, the traditional methods to detect periodontitis only tell us the history of the disease, and not the current status or its future direction$^{18}$. We also cannot use these methods to predict who will suffer from the disease or detect it at its earliest stage, when tissue destruction is just beginning.

Researchers have begun to examine if measuring cytokines in gingival crevicular fluid (GCF) may help diagnose the current condition of periodontal disease or orthodontic root resorption. Extracting GCF from a patient is as simple as inserting a paper point gently into the gingival sulcus. Multiple studies have used GCF as a way to measure cytokine levels. Yamaguchi employed GCF samples to measure levels of cytokines in orthodontic therapy and argues that analysis of GCF samples “may provide
better understanding of the biochemical processes associated with tooth movement, potentially helping clinicians make therapeutic choices based on qualitative and quantitative information” 17. A recent study used a multiplex bead immunoassay and examined 40 cytokines and chemokines in GCF samples of patients with periodontitis; 27 of the 40 cytokines were detected, and 16 of those were significantly different between diseased and healthy patients. The authors stated that GCF samples may be able to describe the current state of disease and perhaps predict its progression 61. Others point out that analysis of cytokine levels in GCF may detect inflammatory lesions in implants at the earliest stages, before they become clinically detectable 16. Suzuki asserts that biochemical analysis of GCF is an effective, non-invasive means of assessing the host response in periodontitis and may be able to illuminate the current state of the disease 18. An interesting look into the possible future of using GCF was a study testing MMP-8 levels in periodontal patients at chair side 71. Examining cytokines in GCF may potentially also be used for any inflammatory process, including trauma.

As described in detail earlier, trauma to the teeth causes an inflammatory response and the release of cytokines. Similar to those treating periodontitis, clinicians are limited in their ability to describe the current state of healing or predict if the tooth will eventually heal or develop problems. Clinical signs of pulpal necrosis may also be signs of healing. Often by the time it is obvious the tooth is necrotic or suffering from aggressive root resorption, irreversible damage has occurred. In the case of an avulsion or a serious luxation injury, dental professionals are currently unable to predict if the tooth will ankylose, or if ankylosis will be permanent and lead to replacement resorption.
Recent studies have examined cytokine levels in patients that suffered avulsion injuries and found that levels of IL-1β and VEGF were elevated in GCF collected from patients with avulsed teeth that healed compared to those that ankylosed; it was also argued that the patient’s genetic polymorphisms in cytokine expression may account for the difference. However, the author is unaware of any studies that have examined cytokine levels in dental concussion, subluxations, and luxation injuries.

The purpose of this pilot study is to examine the GCF cytokine levels immediately after various types of dental trauma to young permanent anterior teeth. The findings of this study may lead to further research into using cytokines to clarify the current status of the tooth after trauma, to predict healing, and to develop novel treatment options for saving the tooth.

**Hypothesis**

The hypothesis of this study is that traumatized teeth will show significant differences in the levels of cytokines and chemokines compared to a control tooth in the same patient.

**Objectives**

1. Examine the levels of 27 biomarkers in the gingival crevicular fluid (GCF) in teeth that have suffered traumatic injuries.

2. Examine the levels of 27 biomarkers in the GCF of an adjacent, non-injured, control tooth in the same arch of the patient.
3. Compare levels of 27 biomarkers in the GCF in teeth that suffered displacement injuries (>1mm) to those teeth that suffered no displacement (<1mm).
Chapter 2: Materials and Methods

Subject selection and study design: The research design was approved by the Nationwide Children’s Hospital Institutions Review Board (IRB). Subjects between the ages of six to twenty who presented to the Nationwide Children’s Dental Clinic or Emergency Department with trauma to anterior permanent teeth were recruited for the study.

Individuals meeting the initial study criteria and their legal guardian were presented with information about the details of the study and asked to sign an informed consent following the Nationwide Children’s Hospital IRB protocol. Data on patient age, ethnicity, sex, medical status, and demographic were obtained. Any traumatized permanent maxillary anterior teeth were considered for the study, but patients were excluded if they were current smokers, had previous trauma to the tooth, infection in the area of trauma, history of diabetes, HIV, blood disorders or immunosuppressive therapy.

Sample collection: All treatment was performed by pediatric dental residents at Nationwide Children’s Hospital dental clinic as part of the routine treatment protocol for traumatized teeth. The sequence of treatment or management approach did not change if the patient was enrolled in the study – the only difference was that at the end of treatment samples of gingival crevicular fluid was collected. Patients were initially treated at either the Nationwide Children’s Dental Clinic or the Emergency Department. Treatment was
based on the International Association of Dental Traumatology (IADT) guidelines depending on the dental trauma sustained. The injured tooth or teeth were isolated with cotton rolls, the supragingival plaque gently removed, and three PerioPaper (Oraflow NY) strips were placed one at a time in the sulcus of each injured tooth until resistance was felt to collect GCF. Three strips were also collected from another, non-injured tooth in the same arch as a control. The strips remained in the sulcus for at least 20 seconds and were then placed in a sterile vial and frozen at minus 50 degrees Fahrenheit. Strips saturated with blood were discarded and replaced with clean ones.

The type of injury to the tooth/teeth were recorded and grouped as follows:

1) Injured teeth that were displaced less than 1mm: concussion, subluxation, Class I, Class II, and Class III fractures.

2) Injured teeth that were displaced more than 1mm: lateral luxation, extrusion, intrusion, and avulsions.

Initially, over 50 patients were enrolled in the study, but the GCF samples were collected at widely disparate times, from within one hour to weeks after injury. To standardize the results and determine a baseline cytokine level of recent trauma, only subjects who had GCF collection within 24 hours of injury were ultimately included. A final number of 34 patients, ages six to twenty, participated in the study, with a total of 48 injured teeth and 34 controls. Injuries included the following: four concussions, four subluxations, one Class I fracture, twenty-three Class II fractures, five Class III fractures (with pulpal exposure), four lateral luxations, two intrusions, one extrusion, and four
avulsions. All teeth in the study had GCF samples collected within 24 hours of the injury.

**Cytokine analysis:** Periopaper strips were thawed on ice and the gingival crevicular fluid was eluted by adding 200 μl of elution buffer containing 50mM Tris/HCl with 5mM CaCl₂, 0.2 NaCl pH 7.6, 1mg/L antipain, 1 mg/L aprotinin, 1 mg/L leupeptin, 125 mg N-ethylaleimide and 50 mg Zwittergent 3-12. The solution was vortexed vigorously at fifteen-minute intervals for an hour. Cytokine analysis was completed using a commercially available multiplexed bead-based assay designed to quantitate multiple cytokines. A panel of 27 cytokines were selected, including: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, FGF-Basic, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α and VEGF.

To briefly describe cytokine analysis, 27 distinct sets of fluorescently dyed beads (Bio-rad laboratories, Inc., Hercules, CA) were conjugated with monoclonal antibodies specific for each cytokine and incubated with 50μl of GCF. 25μl of biotinylated detection antibody and 50μl of Streptavidin-phycoerythrin reporter were sequentially added. The level of each cytokine was analyzed by measuring the fluorescence of each bead type as well as the fluorescent signal from the reporter on a Bio-Plex 200 flow cytometric detection system.
**Data normalization and statistical analysis**: Cytokine levels were measured for each injured and control tooth. From a total of 34 patients, there were 48 injured teeth and 34 controls. The mean value of each of the 27 different cytokines was calculated for the injured teeth and the controls, and each cytokine mean from the injured teeth was paired with the controls. A parametric statistical analysis was used, which assumes that the difference between the pairs is normally distributed, allows for power analysis, and is effective for smaller sample sizes. A paired t-test allows comparison of means to determine if the difference is statistically significant, which is appropriate for our data. Paired t-tests were used to compare the means between the injured and control teeth and to calculate a p-value. The injured teeth were grouped based on the amount of displacement caused, either non-displaced or displaced. Injuries that cause minimal to no displacement (concussion, subluxation, Class I, Class II and Class III fractures) were placed in the non-displaced group, and injuries that caused displacement in the socket (luxation, extrusion, intrusion and avulsion) were placed in the displaced group. Paired t-tests were used for each group. Using the t-tests, we were able to determine if the difference between cytokine levels in injured teeth were statistically significant based on a p-value of <0.05. All statistical analyses were carried out with JMP (SAS Institute Inc., Cary, NC).
Chapter 3: Results

Thirty-four patients were enrolled in our study, ages six to twenty, with a total of 48 injured teeth and 34 controls. All had trauma to the anterior permanent teeth, but the injury ranged from a mild concussion to a complete avulsion of the tooth. A table of the injuries is listed below:

<table>
<thead>
<tr>
<th>INJURY</th>
<th># OF TEETH</th>
<th># OF PATIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concussion/Subluxation alone</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Class I and Class II fractures</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>Class III fractures (pulp exposed)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Luxation and Extrusion</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Intrusion</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Avulsion</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5. Injuries in study

Due to the limited numbers of different types of trauma in our study, we were unable to examine and compare distinct types of trauma. Recognizing the limitations of such a general distinction, we classified the teeth by “Displaced Injuries” or “Non-Displaced.” A non-displaced injury included any trauma that caused minimal (<1mm) of
movement to the tooth: concussion, subluxation, Class I, Class II, and Class III fractures.

There were 37 non-displaced injuries. A displaced injury caused moderate to severe (>1mm) of displacement of the tooth in the socket: luxation, extrusion, intrusion, and avulsion injuries were combined into this group. There were 11 displaced injuries. The levels of significant cytokines are listed below:

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>&lt;1mm</th>
<th>&gt;1mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF</td>
<td></td>
<td>*** - Injured</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td>*** - Injured</td>
</tr>
<tr>
<td>IL-1ra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td></td>
<td>** - ONLY injured</td>
</tr>
<tr>
<td>IL-4</td>
<td>* - control</td>
<td>** - ONLY injured</td>
</tr>
<tr>
<td>IL-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>*** - injured</td>
<td>*** - injured</td>
</tr>
<tr>
<td>IL-7</td>
<td></td>
<td>*** - control</td>
</tr>
<tr>
<td>IL-8</td>
<td>** - Injured</td>
<td>** - injured</td>
</tr>
<tr>
<td>IL-9</td>
<td>*** - control</td>
<td>** - control</td>
</tr>
<tr>
<td>IL-10</td>
<td>*** - injured</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td>*** - control</td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>*** - ONLY injured</td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>*** - control</td>
<td></td>
</tr>
<tr>
<td>FGF Basic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-CSF</td>
<td></td>
<td>** - Injured</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>*** - control</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>*** - injured</td>
<td>*** - injured</td>
</tr>
<tr>
<td>IP-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>** - control</td>
<td>** - control</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>** - injured</td>
<td></td>
</tr>
<tr>
<td>MIP-1β</td>
<td>** - injured</td>
<td>** - injured</td>
</tr>
<tr>
<td>RANTES</td>
<td>** - injured</td>
<td>*** - injured</td>
</tr>
<tr>
<td>TNF-α</td>
<td>*** - control</td>
<td>*** - ONLY control</td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td>** - Injured</td>
</tr>
</tbody>
</table>

Table 6. Significant cytokines found in study
Comparison of cytokine levels in “Displaced Injuries” to control teeth yielded significant differences. In teeth that were displaced by more than 1mm, a significantly greater level \((p<0.001)\) was found in the amounts of PDGF, IL-1\(\beta\), IL-6, IFN-\(\gamma\), MIP-1\(\beta\), RANTES, IL-2, IL-4, IL-8, G-CSF, and VEGF compared to control teeth. The displaced teeth also had significantly significant lower levels of IL-7, IL-9, MCP-1 and TNF-\(\alpha\) compared to controls. We were unable to detect levels of TNF-\(\alpha\) in any of the injured teeth, and levels of IL-2, IL-4, Eotaxin, and MIP-1\(\alpha\) were not found in control teeth. IL-13, IL-15, IL-17, and GM-CSF were not detected in any teeth in this group.

Teeth that did not suffer a displacement injury, the “Non-Displaced Injuries,” also differed in cytokine levels compared to controls. Injured teeth had significantly higher levels of IL-6, IL-8, IL-10, IL-17, IFN-\(\gamma\), MIP-1\(\alpha\), MIP-1\(\beta\), and RANTES compared to controls \((p<0.001)\). Injured teeth had lower levels of IL-4, IL-9, IL-15, Eotaxin, GM-CSF, MCP-1, and TNF-\(\alpha\) \((p<0.01)\). IL-17 was not detected in any control teeth.

Both groups of injured teeth had significantly higher levels of IL-6 \((p<0.0001)\), IL-8 \((p<0.001)\), IFN-\(\gamma\) \((p<0.0001)\), MIP-1\(\beta\) \((p<0.001)\), and RANTES \((p<0.001)\). They also had lower levels of IL-9, MCP-1, and TNF-\(\alpha\).

In contrast to non-displaced teeth, the displaced injuries had higher levels of PDGF, IL-1\(\beta\), IL-4, G-CSF, and VEGF compared to controls, while they had lower levels of IL-7. Non-displaced teeth had higher levels of IL-10, IL-17 and MIP-1\(\alpha\) compared to displaced injuries, and lower levels of IL-15, Eotaxin, GM-CSF, and IL-4. Interestingly, IL-4 was significantly lower in non-displaced teeth, but was significantly higher in displaced teeth compared to controls.
Figure 1. Cytokines in non-displaced injuries

Figure 2. Cytokines in displaced injuries
Chapter 4: Discussion

Due to our limited number of study samples, the injured teeth were categorized as “displaced” or “non-displaced”. To be practical for the dental clinician, a “displaced” injury refers to a clinically obvious displacement of the tooth in the socket. Luxation, extrusion, intrusion and complete avulsion injuries were included in this category. Traumatic injuries that caused minimal movement of the tooth in the socket, such as concussion or subluxation injuries and fractures, were grouped in the non-displaced category. Concussion and subluxation injuries may damage the periodontium, but by definition the tooth is not re-positioned in the socket by any measurement a dentist would detect clinically.

Analyzing the gingival crevicular fluid of traumatized teeth may become a practical tool to assist dentists’ treatment decisions for dental trauma. An immature or young permanent tooth can be very difficult to treat. The clinician will want to delay endodontic therapy as long as possible to allow the tooth to develop, but the tooth will suffer rapid root resorption if it becomes necrotic. The difficulty lies in knowing when a tooth is healing or when it is necrotic, since signs of healing can mimic those of necrosis. In recent years, much attention has been directed toward the role of cytokines in all aspects of healing throughout the body and their role in pathology. Cytokines have been associated with inflammatory diseases such as rheumatoid arthritis, diabetes, asthma, and osteoporosis. If cytokines impact healing throughout the body, it is likely that they can
influence healing of dental trauma. As shown in periodontal and orthodontic literature, GCF can be analyzed to determine the presence and amount of cytokines around teeth\textsuperscript{17, 18, 61}. Certain cytokines have been associated with periodontitis, inflammatory bone destruction, and root resorption during orthodontic movement. As several authors have pointed out, GCF may be able to illuminate the \textit{current} status of the periodontium, something that other clinical and radiographic signs and symptoms cannot do\textsuperscript{18, 61}. Collecting GCF is simple, non-invasive, and can be done in a couple of minutes. Analyzing GCF of traumatized teeth may help reveal the current state of injured teeth, and may even be able to predict future healing.

**Significant cytokine levels in both displaced and non-displaced injuries:**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Injured Teeth (Both displaced and non-displaced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Elevated</td>
</tr>
<tr>
<td>IL-8</td>
<td>Elevated</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Elevated</td>
</tr>
<tr>
<td>RANTES</td>
<td>Elevated</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Decreased</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Decreased</td>
</tr>
<tr>
<td>IL-9</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

Table 7. Significant cytokine levels in both displaced and non-displaced injuries

This pilot study found that there are higher levels of **IL-6** (p<0.0001), **IL-8** (p<0.001), **IFN-γ** (p<0.0001), **MIP-1β** (p<0.001), and **RANTES** (p<0.001) in both displaced and non-displaced injured teeth compared to controls. The injured teeth also had significantly lower levels of **IL-9**, **MCP-1**, and **TNF-α**.
After injury, injured cells and tissues release cytokines that initiate acute inflammation and attract neutrophils and macrophages. IL-6, IL-8, IFN-γ, MIP-1β and RANTES all participate in this process. Below is a list of the significant cytokines found in both displaced and non-displaced teeth and their clinical significance.

**IL-6**: Interleukin-6 cytokine was found in elevated levels in both displaced and non-displaced teeth. IL-6 is considered both a pro-inflammatory and anti-inflammatory mediator. Released by macrophages, T-cells, fibroblasts, osteoblasts and others, one of its chief functions is to stimulate acute inflammation in damaged tissue\(^{26,29}\). In early inflammation it attracts neutrophils and macrophages to the injury, which is critical for healing. IL-6 synergizes with TGFβ to increase levels of TH cells that produce IL-17, which also increases inflammation. As an anti-inflammatory agent, it inhibits the effects of IL-1 and TNF-α, and activates other anti-inflammatory mediators IL-1ra and IL-10 \(^{35}\). IL-6 also assists with the proliferative phase of healing by inducing angiogenesis and granulation tissue formation \(^{34}\). Mice that are IL-6 deficient show delayed healing \(^{73}\). However, if IL-6 levels continue to stay elevated throughout the healing process, prolonged inflammation occurs \(^{34}\). IL-6 promotes osteoclastogenesis, contributes to bone loss, and has been found in high levels in subjects with periodontitis, peri-implantitis, and osteoporosis \(^{60}\). The cytokine has also been detected in the GCF of teeth that are under orthodontic forces that cause bone remodeling and root resorption \(^{52}\). In injured teeth, IL-6 is released to initially stimulate inflammation and then to assist with angiogenesis and healing. However, if the levels continue to stay elevated, the expectation is for the injured tooth to suffer periodontal destruction, root resorption, and delayed healing.
**IL-8**: Interleukin-8 was detected in significantly higher levels in injured teeth. Released by macrophages and epithelial cells, IL-8 is a chemokine in the CXC family that is important in initial inflammation. IL-8 is also known as “neutrophil chemotactic factor,” attracts neutrophils to sites of infection and injury, and triggers the neutrophils to become phagocytic once they arrive. However, it also helps with angiogenesis and tissue remodeling. Along with IL-6, IL-8 stimulates osteoclasts and contributes to bone resorption by stimulating more production of RANKL. High levels of IL-8 in GCF have been associated with gingivitis, periodontitis, and heavy orthodontic forces. IL-8 mirrors IL-6 by stimulating acute inflammation and later helping with healing. However, elevated levels over time may indicate chronic inflammation and contribute to bone destruction of injured teeth.

**IFN-γ**: Interferon-gamma was found in higher levels in traumatized teeth. The principal function of the cytokine is activation of macrophages, and is produced by activated T cells and natural killer cells. Macrophages phagocytize foreign and necrotic material, but can also contribute to widespread tissue destruction by releasing cytotoxic substances. By being a potent activator of macrophages, IFN-γ may indirectly contribute to tissue damage, including bone resorption. However, studies give conflicting results about the role of IFN-γ in bone metabolism in vivo. Several studies indicate that IFN-γ directly inhibits osteoclastogenesis. Knockout mice without IFN-γ have a reduction in bone volume, while mice given IFN-γ had a marked increase in bone strength. Other research links bone resorption with IFN-γ, and assert that IFN-γ increases levels of TNF-α and IL-1, which are potent activators of osteoclasts.
study argues that IFN-γ may actually promote and inhibit bone resorption depending on the amount of the cytokine in the injured area. They found high levels of IFN-γ stimulate osteoclasts, while low levels inhibit bone loss via osteoblast activation\textsuperscript{76}. The cytokine has been found in higher levels in patients with periodontitis\textsuperscript{42,49}. The presence of elevated IFN-γ in injured teeth signals acute inflammation, and that macrophages are being recruited and activated. Although the role of IFN-γ in bone remodeling is still being debated, low levels may promote bone formation. Low levels over an extended time may indicate the injured tooth is resisting damaging bone resorption, while high levels would indicate chronic bone destruction.

**MIP-1β:** Macrophage Inflammatory Protein-1β chemokine, also known as CCL-4, was found in significantly higher levels in both displaced and non-displaced teeth. The chemokine is closely related to MIP-1α, which plays an essential role in healing by attracting macrophages and TH-1 lymphocytes to acute inflammation\textsuperscript{41}. A decreased level of MIP-1 reveals a diminished ability to recruit macrophages which negatively impacts wound healing\textsuperscript{80}. However, macrophages and TH-1 cells are also sources of pro-inflammatory and bone resorbing cytokines. MIP-1β itself functions to recruit osteoclasts to new sites and promotes their survival. Studies have shown cutaneous wounds have higher levels of MIP-1 over time\textsuperscript{37}. MIP-1β is also higher in tissues suffering from periodontal disease, and is highly expressed in bone remodeling during orthodontic movement\textsuperscript{48,59}. While MIP-1β is critical in recruiting macrophages to begin the healing process, it also promotes bone resorption. In traumatized teeth, MIP-1β
would initially assist in cleaning the area of necrotic cells and bacteria, but prolonged levels signal a high potential for periodontal destruction and a poorer prognosis.

**RANTES**: The RANTES chemokine was found in higher levels in both groups of traumatized teeth. Is also known as CCL-5, and is an important mediator of acute and chronic inflammation\(^{39}\). RANTES is closely linked to MIP-1\(\alpha\) and MIP-1\(\beta\). Like MIP-1, it is released in areas of inflammation and attracts macrophages and leukocytes to the injury\(^{41,48,81}\). MIP-1 is implicated as being essential for continued levels of RANTES, and our study also found RANTES and MIP-1\(\beta\) to both be significantly higher in injuries\(^{80}\). In a study examining levels of cytokines in inflamed knee joints, including after a traumatic event, elevated levels of RANTES were detected\(^{39}\). The periodontal literature reveals elevated levels of RANTES in untreated periodontal patients, and high levels are associated with chronic periodontitis\(^{58,60}\). Researchers have found that even after treatment, RANTES remained high in patients with periodontal disease, and suggest that elevated RANTES may be an intrinsic factor in patients with periodontitis\(^{58}\). RANTES has also been associated with bone and root resorption during orthodontic movement\(^{50}\). Like MIP-1, RANTES recruits osteoclasts to injured areas and promotes bone resorption\(^{48}\). Continued high levels of RANTES in traumatized teeth would imply chronic inflammation and delayed healing, and likely periodontal destruction.

**MCP-1**: Monocyte Chemoattractant Protein-1 was found in decreased levels in traumatized teeth. Also known as CCL-2, this chemokine recruits monocytes and dendritic cells to inflammation. The presence of monocytes and macrophages in the wound area is critical, since they are responsible for wound healing and releasing
important cytokines. In one study, knockout mice without MCP-1 showed impaired healing, and a decrease in essential cytokines such as IL-1β, IL-10 and TNFα\textsuperscript{82}. Studies of MCP-1 deficient mice also reveal higher levels of RANTES, MIP-1α and MIP-1β, indicating that MCP-1 inhibits their release\textsuperscript{82}. Our study supports the finding that low levels of MCP-1 are associated with higher levels of MIP-1β and RANTES. While MCP-1 is crucial for healing, it also stimulates osteoclast differentiation, guides osteoclasts to areas of injury, and promotes their survival – this fosters bone resorption\textsuperscript{35, 50}. It has been associated with joint destruction during rheumatoid arthritis, as well as bone and root resorption under heavy orthodontic loading\textsuperscript{50, 62}. Our study reinforces previous findings that lower levels of MCP-1 are associated with higher levels of RANTES and MIP-1β.

**IL-9:** Both displaced and non-displaced teeth have lower levels of Interleukin-9 in relation to controls. IL-9 is released by neutrophils, T-cells, mast cells and others. It stimulates cell division and growth. IL-9 is being studied for its possible role in inflammatory diseases, allergies, and asthma\textsuperscript{83}.

**TNF-α:** Closely associated with and similar to IL-1, Tumor Necrosis Factor-alpha was found in lower levels in our study. TNF-α is a potent inflammatory cytokine that stimulates and is intimately involved with acute inflammation\textsuperscript{42}. It recruits neutrophils and macrophages and fuels other cells to release chemotactic chemokines, such as RANTES, and pro-inflammatory cytokines like IL-6, IL-8 and IL-1\textsuperscript{84}. It promotes osteoclastogenesis directly and has been shown to trigger root resorption in rats\textsuperscript{27, 52, 67}. Studies also suggest TNF-α is associated in periapical infections that cause bone
resorption around the root of the tooth. The presence of TNF-α indicates a strong acute inflammatory response, and continued levels are associated with pathology and chronic inflammation. It is surprising that levels of TNF-α would be lower in traumatized teeth; in teeth displaced over 1mm there were no levels detected at all in injured teeth. This may indicate that other cytokines are involved in dental inflammation due to trauma.

In summary, both groups of injured teeth had significantly higher levels of known inflammatory cytokines, which is not surprising. High levels of IL-6, IL-8, and INF-γ show the presence of acute inflammation and the recruitment of neutrophils and macrophages that release more inflammatory cytokines. Even teeth that were not displaced had high levels of IL-6 and IL-8, which stimulate inflammation but are also important in angiogenesis. This may indicate that even concussion and subluxation injuries can cause significant damage to surrounding bone and blood supply.

The presence of these cytokines is important for early wound healing, but can cause chronic inflammation and pathology over time. MIP-1β and RANTES assist in recruiting macrophages, which are important to start the process of wound repair; but they also attract osteoclasts. The influx of osteoclasts is normal in the early stages of healing, since they help with remodeling and repair of damaged bone. Prolonged elevation of these cytokines would suggest chronic inflammation and the potential for pathologic bone resorption, which may be an early indicator of impaired healing. It is interesting to note the high levels of IL-6 and IFN-γ, which have both pro- and anti-inflammatory properties. IL-6 and IFN-γ at first enhance inflammation, but then may help dampen the inflammatory response and bone destruction over time. The continued
presence of low levels of IL-6 and IFN-γ may be an indicator of future healing. Both sets of injured teeth had a significantly lower level of TNF-α, which is an important cytokine for acute inflammation; this is an unexpected result. For example, studies have found higher levels of IL-1, IL-6, and TNF-α for the first few days after a bone injury\(^4\).
inflammation. Incidentally, IL-10 was found to be greater in injured teeth, but not to a significant level compared to controls. IL-4 assists with wound healing by stimulating fibroblast proliferation, and also inhibits osteoclasts and bone resorption. IL-4 is also important in repairing vascular damage by stimulating the release of VEGF, which will be discussed below. The presence of IL-4 in displaced teeth signals an attempt to limit damaging inflammation and control pathologic bone resorption. Continued levels of IL-4 may indicate a promising sign that the tooth is healing and resisting periodontal destruction that could cause damaging consequences.

**PDGF**: Platelet-Derived Growth Factor was detected in higher levels in displaced injuries. PDGF is involved in each stage of wound healing. After injury, platelets release several cytokines and growth factors, including PDGF and IL-1 that attract neutrophils. As macrophages arrive to the injured area, they release more PDGF. Working with other growth factors, PDGF helps organize a scaffold for fibroblasts to infiltrate and repair damaged tissue. Increased levels of PDGF are associated with an increase in VEGF, which was also found in our study. As the wound heals, PDGF is important in blood vessel development and maturation, and is critical for the formation of granulation tissue and the repair of damaged vasculature in the periodontium. Through a complex relationship, TNF-α is reduces the levels of PDGF, and, as described above, TNF-α was found to be significantly lower in injured teeth. In chronic wounds, levels of PDGF have found to be low, and recombinant human PDGF is currently the only FDA approved drug for chronic wound treatment. PDGF has been used to treat patients with periodontal bony defects and increase osseointegration of dental implants. PDGF
also promotes the proliferation of dental stem cells that assists with healing of the pulp after injury or infection\textsuperscript{87}. A significantly higher level of PDGF is expected in teeth that were displaced, since the blood supply to the tooth is damaged. The presence of PDGF shows that the injured periodontium and vasculature are in early stages of repair and angiogenesis. As damaged blood vessels are repaired, the potential of revascularization of a displaced tooth is higher. Thus, continued elevated levels of PDGF should be a promising sign that the tooth is healing and continued vitality of the pulp is more likely.

**VEGF**: Vascular Endothelial Growth Factor is a companion to PDGF, and was also found in significantly higher amounts in displaced teeth. Like PDGF it stimulates angiogenesis. VEGF is released by several cell types, including platelets, neutrophils, endothelial cells and macrophages. The chief stimulus for VEGF release from injured tissue is hypoxia or ischemia. VEGF functions to create granulation tissue and new blood vessels after a traumatic injury and is important in the early events of angiogenesis by promoting endothelial cell migration and proliferation\textsuperscript{23}. IL-4, PDGF and IL-1β stimulate cells to increase secretion of VEGF\textsuperscript{22,62}. In animal studies, VEGF has been found to encourage angiogenesis in diabetic ischemic limbs and improve the healing of diabetic wounds by promoting blood vessel formation\textsuperscript{23}. VEGF also recruits and promotes dental stem cells that help with the healing process\textsuperscript{87}. In a recent pilot study examining cytokine levels in ankylosed teeth, higher levels of VEGF were found in teeth that healed compared to those that ankylosed\textsuperscript{72}. Like PDGF, elevated levels of VEGF indicate damage to the vasculature of a traumatized tooth, and elevated levels over time may indicate healing of the injured periodontium.
**IL-1β**: While the level of Interleukin-1 beta was higher in non-displaced teeth, it was not significant. In displaced teeth, IL-1β was significantly greater. It is released by neutrophils, macrophages and gingival fibroblasts. This cytokine is recognized as a powerful inflammatory mediator that initially attracts neutrophils, and is critical in stimulating the innate, or acute, host response\(^4^2\). Pro-inflammatory macrophages release high amounts of IL-1β that attracts more leukocytes. IL-1β is believed to increase expression of IL-6, IL-8, RANTES, and other inflammatory mediators during early wound repair\(^8^4\). If the level of IL-1 remains high over one week after injury, chronic inflammation and bone destruction develops\(^4^1\). Prolonged expression of IL-1β is connected with inflammatory diseases throughout the body, including rheumatoid arthritis and asthma\(^4^2, 4^8, 8^8\). IL-1 has also been consistently associated with gingival inflammation, chronic periodontitis, bone destruction, and root resorption\(^4^0, 4^8, 6^0\). The cytokine also decreases the ability of dental pulp stem cells to help with healing\(^8^7\). IL-1β elevation reveals an acute inflammatory response in displaced teeth, which is essential in early wound repair. However, continued high levels of IL-1 imply destructive, prolonged inflammation that may indicate diminished healing of an injured tooth.

**G-CSF**: Granulocyte colony-stimulating factor is also elevated in displaced teeth. G-CSF is produced by many cells to stimulate the bone marrow to create more white blood cells, particularly neutrophils\(^8^9\). It is one of the chief cytokines responsible for mobilizing neutrophils from the bone marrow to areas of acute inflammation\(^9^0\). In many ways, it may be considered an anti-inflammatory mediator since it stimulates repair of injury. Current research has found that G-CSF is important for tissue protection and
repair outside the blood system. G-CSF has been found in neural, liver and cardiac tissue after brain injury, spinal cord disease, liver damage, and heart disease\textsuperscript{91}. The presence of this factor in dental trauma represents an effort to increase leukocytes to combat infection and may also have a protective and reparative function.

**IL-7:** Interleukin-7 was detected in significantly lower amounts in injured teeth. IL-7 is released by T and B cells and is considered a hematopoietic growth factor. It also is important in T cell homeostasis. IL-7 is associated with chronic periodontitis\textsuperscript{63}.

In summary, displaced teeth had uniquely significant elevated levels of several cytokines. Displacement injuries damage the periodontal support of the tooth; the bone and PDL are traumatized and the tooth’s blood supply is damaged or even completely destroyed. A severe injury seems to stimulate an increase in IL-1β to initiate inflammation important to healing, but at the same time IL-4 is released to limit the inflammatory response. IL-4 stimulates the release of several anti-inflammatory mediators, and promotes growth factors that will initiate angiogenesis. PDGF and VEGF promote angiogenesis, dental stem cell proliferation, and deter chronic inflammation, which is critical for healing of the injured vasculature and to ensure continued vitality of the pulp. Continued high levels of IL-1β over time, with a decrease in IL-4, PDGF and VEGF may indicate lack of healing.
Significant cytokines in only non-displaced injuries (<1mm)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Injured teeth (non-displaced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>Elevated</td>
</tr>
<tr>
<td>IL-17</td>
<td>Elevated</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Elevated</td>
</tr>
<tr>
<td>IL-15</td>
<td>Decreased</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>Decreased</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Decreased</td>
</tr>
<tr>
<td>IL-4</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

Table 9. Significant cytokines in non-displaced injuries

Thirty-seven teeth in our study sustained injuries that did cause displacement. Injuries include concussion, subluxation, and crown fractures. Minimal damage to the periodontium and vascular supply occurred. Non-displaced injuries revealed elevated levels of **IL-10** (p<0.0001), **IL-17** (p<0.0001), and **MIP-1α** (p<0.001), while **IL-15** (p<0.001), **Eotaxin** (p<0.0001), **GM-CSF** (p<0.0001) and **IL-4** (p<0.01) were lower.

**IL-10**: Interleukin-10 was elevated in non-displaced injuries. IL-10 inhibits inflammation by repressing the expression of other pro-inflammatory cytokines. For example, IL-10 inhibits production of IL-1β, IL-6, TNF-α, and is also associated with lower levels of IL-4\(^{26}\). In our study, levels of IL-1β, TNF-α, and IL-4 were also decreased, which may be explained by the high levels of IL-10. The anti-inflammatory cytokine has been studied as a therapy to combat asthma, allergies, and other hyperactive immune responses\(^{29}\). However, a burn study found that elevated levels of IL-10 were associated with a higher incidence of sepsis\(^{38}\). Thus, while IL-10 limits damaging inflammation, high levels can also negate some of the beneficial attributes of
inflammation, such as fighting infection. The presence of IL-10 in non-displaced teeth may indicate a more muted inflammatory response compared to displaced teeth, and should be seen as a positive sign that the site is limiting destructive damage from prolonged inflammation.

**IL-17:** Interleukin-17 was found to be significantly higher in non-displaced teeth. Research is still being done on the effects of IL-17, but it is considered a pro-inflammatory cytokine that induces the expression of several other cytokines such as IL-6, G-CSF, GM-CSF, and IL-8\textsuperscript{26,88}. It also helps stimulate production of neutrophils and to direct them to inflammation\textsuperscript{88}. Only non-displaced teeth had higher levels of IL-17, and no control teeth registered any amount of the cytokine. Our study’s results support that elevated IL-17 is associated with elevated IL-6 and IL-8, but there was not an increase in G-CSF or GM-CSF in non-displaced injuries. Chronic high levels of IL-17 are associated with asthma, inflammatory bowel disease and multiple sclerosis\textsuperscript{26}. IL-17 is elevated in rheumatoid arthritis synovial fluid and has been found to exacerbate the disease\textsuperscript{88}. Periodontal studies are conflicted about the role of IL-17 in periodontitis. Some find IL-17 linked to implant periodontitis and chronic periodontitis\textsuperscript{65,92}. Others suggest that it may actually protect the supporting structures of the teeth because of its ability to attract neutrophils that combat oral pathogens\textsuperscript{42,88}. More research is needed to define the function of IL-17 in inflammation, especially when associated with infection.

**MIP-1α:** Levels of Macrophage Inflammatory Protein-1alpha (CCL-3) were higher in non-displaced teeth. During the acute inflammatory stage, MIP-1α is released to attract and activate neutrophils. It also recruits macrophages to the injury, and
promotes osteoclast activity. Due to its activation of osteoclasts, MIP-1α is linked to bone resorption during orthodontic movement\textsuperscript{50}. It is also associated with diseased periodontium\textsuperscript{59,63}. The presence of MIP-1α in non-displaced injuries signal that inflammation has begun and macrophages are being recruited to the injured area. Prolonged levels of MIP-1α should be seen as a negative indicator of healing.

**IL-15**: Interleukin-15 was significantly lower in non-displaced teeth compared to controls. IL-15 is similar in structure to several other cytokines, including IL-2, IL-4, IL-7 and IL-9. While it defends the host from infection, high levels have been associated with dysregulation of the immune system\textsuperscript{93}. IL-15 is implicated in celiac disease, inflammatory bowel disease, psoriasis, and rheumatoid arthritis\textsuperscript{94}. A pilot study examined biomarkers in the socket irrigant and pulpal tissue of avulsed teeth and found that IL-15 was significantly higher in teeth that eventually ankylosed\textsuperscript{95}. More research is needed to determine its function in inflammation, especially in the oral cavity.

**GM-CSF**: Granulocyte macrophage colony-stimulating factor was decreased in non-displaced teeth. The protein is produced by fibroblasts, macrophages, T cells and others to stimulate the bone marrow to create more white blood cells. It is essential in the initial inflammatory stage of wound healing because it increases the number of neutrophils and enhances their function at the injured area\textsuperscript{23}. It is unclear why levels of GM-CSF would be lower in non-displaced teeth compared to controls.

To summarize, non-displaced injuries demonstrated higher levels of IL-10. IL-10 is an important anti-inflammatory cytokine, and perhaps a mild injury that does not significantly damage the periodontium triggers a milder inflammatory response.
However, levels of IL-4 were lower. Studies have associated higher levels of IL-4 with more production of IL-10, but this study did not support that connection. Another intriguing result is that GM-CSF was lower in injured non-displaced teeth and completely absent in displaced teeth.

Our study suffered from several limitations. A major limitation is the sample size, which depended completely on the number patients presenting in the emergency department or dental clinic for initial trauma. Due to the stress and pain of injury, some patients were not cooperative for treatment and collection of GCF was deferred. Trauma that displaced the teeth was also commonly associated with gingival lacerations or significant bleeding, and non-contaminated GCF samples were not able to be collected. Another limitation is control teeth were chosen in the same arch as the injured tooth. There is a possibility that the control tooth could have sustained a slight injury without being detected by the researcher.
Chapter 5: Summary and Conclusions

In summary, within twenty-four hours cytokine levels in traumatized teeth are significantly different than in control teeth. Both displaced and non-displaced teeth registered high levels of pro-inflammatory cytokines. Displaced teeth also had significantly higher levels of anti-inflammatory cytokines and growth factors that promote angiogenesis.

Gingival crevicular fluid samples were obtained within twenty-four hours of the traumatic event; in many patients the samples were collected within hours. Thus, the cytokines found in this study represent a “baseline” level after a dental injury. The cytokines were stimulated by the injured cells and tissue in the local environment. As more time passes, a swarm of inflammatory cells such as leukocytes, macrophages, and lymphocytes will arrive to the injured area and the cytokine levels will change. The presence of bacteria will also stimulate production of cytokines. The balance between healing and damaging inflammation will continually change, with cytokines driving the process. If the scale is tipped towards chronic inflammation, the tooth will likely sustain long-term, even permanent, damage. Unfortunately, the dental clinician has limited ability to determine the current healing state of the tooth or to predict healing. Analyzing gingival crevicular fluid may be a powerful tool to determine the status of an injured tooth.
Future studies can examine the cytokine levels in traumatized teeth over time and correlate those levels to the long-term status of the tooth. Studies with larger sample sizes can also explore the presence of cytokines based on specific dental injuries; for example, the cytokine levels in lateral luxation injuries could be compared to intrusion injuries. Our study shows that cytokines can be detected in traumatized teeth GCF samples, and that levels of certain cytokines were significantly different in injured teeth.
References


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72. Ashley A. Immune mediators in gingival crevicular fluid as predictors of healing outcomes in re-implanted permanent incisors: a pilot investigation.
## Appendix A: Tables

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell Source</th>
<th>Cell Target</th>
<th>Primary Effect(s)</th>
</tr>
</thead>
</table>
| TNF-α (Tumor Necrosis Factor-Alpha) | Macrophages T<sub>H</sub>-1 cells Mast cells | T cells, B cells Endothelial cells | - Important mediator of acute inflammation  
- Recruits neutrophils and macrophages to site of injury  
- Stimulates other cells to release chemotactic chemokines |
| IL-1β                  | Monocytes/Macrophages Fibroblasts Epithelial cells Endothelial cells | T cells, B cells Endothelial cells | - Similar to TNF-α  
- Activation of acute inflammation  
- Activates T-cells |
| IL-1ra (IL-1 receptor antagonist) | T cells B cells | T cells, B cells Epithelial cells | - Anti-inflammatory  
- Binds to IL-1 receptor and blocks IL-1β |
| IL-2                   | T cells NK cells                 | T cells, B cells Monocytes | - Major growth factor for T cells  
- Promotes growth of B cells, activates NK cells and macrophages |
| IL-4                   | T cells (T<sub>H</sub>-2) Mast cells Macrophages | Naïve T cells T<sub>H</sub>-2 cells B cells | - Considered an anti-inflammatory cytokine  
- Stimulates differentiation and growth of T<sub>H</sub>-2 cells  
- Regulates humoral immunity  
- Stimulates B cell class switching  
- Inhibits bone resorption |

Table 10. Table of cytokines.
<table>
<thead>
<tr>
<th>Table 10 continued</th>
</tr>
</thead>
</table>
| **IL-5** | **T_{H-2}** cells | B cells  | Eosinophils | - Stimulates B cell growth and differentiation  
- Activates mature eosinophils  
- May have anti-inflammatory function |
| **IL-6** | T cells (T_{H-2})  
Macrophages  
Fibroblasts | T cells, B cells  
Mature B cells | - Important in activating acute inflammation  
- Stimulates B cell maturation  
- Enhances bone resorption  
- Also has an anti-inflammatory role |
| **IL-7** | Bone marrow stromal cells  
Thymus | T cells  
B cells | - Hematopoietic growth factor  
- Stimulates production of T cells, B cells, NK cells  
- Associated with chronic periodontitis |
| **IL-8** | Macrophages  
Epithelial cells  
Platelets  
Mast cells | Neutrophils | - Attracts neutrophils to injury  
- Stimulates bone resorption  
- Increases with excessive orthodontic force |
| **IL-9** | T cells | T cells, B cells | - Stimulates antibody production  
- T cell growth  
- Implicated in asthma |
| **IL-10** | T cells (T_{H-2})  
Activated macrophages | Macrophages  
T cells | - Anti-inflammatory cytokine  
- Inhibits inflammation  
- Down regulates and dampens inflammatory cytokine production |
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sources</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>Macrophages, NK cells</td>
<td>Naïve T cells, differentiation to Th-1, stimulates production of IFN-γ, low levels associated with less periodontal disease</td>
</tr>
<tr>
<td>IL-13</td>
<td>T cells (Th-2)</td>
<td>T cells, B cells, similar to IL-4, anti-inflammatory, stimulates Th-2 response</td>
</tr>
<tr>
<td>IL-15</td>
<td>Monocytes/Macrophages, Fibroblasts</td>
<td>NK cells, similar to IL-2, induces proliferation of NK cells, often triggered by viral infections</td>
</tr>
<tr>
<td>IL-17</td>
<td>T cells</td>
<td>Macrophages, Fibroblasts, Endothelial cells, Epithelial cells, promotes inflammation, recruits neutrophils and monocytes, promotes production of IL-6, G-CSF, GM-CSF, IL-1, TNF-α, IL-8, MCP-1, implicated in autoimmune diseases like asthma, lupus, rheumatoid arthritis</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>Various cell types</td>
<td>Eosinophils, attracts eosinophils</td>
</tr>
<tr>
<td>FGF Basic (Basic Fibroblast Growth Factor)</td>
<td>Damaged epithelial, endothelial, fibroblast cells</td>
<td>Endothelium, promotes angiogenesis during wound healing</td>
</tr>
<tr>
<td>G-CSF (Granulocyte Colony-stimulating Factor)</td>
<td>Macrophages, Endothelium, T cells</td>
<td>Bone marrow, stimulates bone marrow to produce granulocytes and stem cells</td>
</tr>
</tbody>
</table>

Continued
| **GM-CSF**  
| (Granulocyte macrophage colony-stimulating factor) | **Macrophages**  
| T cells  
| Fibroblasts  
| Endothelium | **Bone marrow**  
| - Stimulates stem cells to produce granulocytes and monocytes |
| **IFN-γ**  
| (Interferon-gamma) | **T cells (T<sub>H</sub>-1)**  
| NK cells | **Monocytes/Macrophages**  
| Endothelial cells | - Important for innate and adaptive immunity  
| - Activates macrophages  
| - Associated with inflammatory and autoimmune diseases  
| - Inhibits osteoclasts |
| **IP-10**  
| (CXCL-10) | **Fibroblasts**  
| Monocytes  
| Endothelium | **Monocytes/Macrophages**  
| T cells | - Secreted by cells after being triggered by IFN-γ  
| - Chemoattraction for monocytes/macrophages, T cells  
| - T cell regulation  
| - Inhibits angiogenesis |
| **MCP-1**  
| (Monocytes chemotactic protein-1/CCL-2) | **Monocytes**  
| T cells | **Monocytes**  
| Basophils  
| Osteoclasts  
| Osteoblasts | - Induced by PDGF  
| - Chemotactic for monocytes and basophils, does not attract neutrophils  
| - Works with RANTES to further osteoclast differentiation |
| **MIP-1α**  
| (Macrophage Inflammatory Protein 1-alpha/CCL-3) | **Macrophages**  
| **Granulocytes**  
| T cells  
| Macrophages  
| Fibroblasts | - Activate neutrophils, eosinophils, basophils  
| - Recruits granulocytes and T cells to injury/inflammation  
| - Induce production of IL-1, IL-6, TNF-α |
| **MIP-1β**  
| (Macrophage Inflammatory Protein 1-beta/CCL-4) | **Macrophages**  
| **Granulocytes**  
| T cells  
| Macrophages  
| Fibroblasts | - Chemotactic for NK cells, monocytes, T cells  
| - Induce production of IL-1, IL-6, TNF-α |

Continued
Table 10 continued

<table>
<thead>
<tr>
<th><strong>PDFG</strong> (Platelet Derived Growth Factor)</th>
<th><strong>Platelets</strong>&lt;br&gt;Endothelial cells&lt;br&gt;Macrophages&lt;br&gt;Smooth muscle cells</th>
<th><strong>Endothelium</strong>&lt;br&gt;Neutrophils</th>
<th>- Angiogenesis&lt;br&gt;- Attracts neutrophils to inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RANTES</strong>&lt;br&gt;(Regulated on Activation, Normal T cell Expressed and Secreted/CC L-5)</td>
<td>T cells&lt;br&gt;Fibroblasts&lt;br&gt;Mast cells</td>
<td>T cells&lt;br&gt;Granulocytes&lt;br&gt;NK cells</td>
<td>- Chemotactic for T cells, granulocytes&lt;br&gt;- Recruits leukocytes to inflammation</td>
</tr>
<tr>
<td><strong>VEGF</strong>&lt;br&gt;(Vascular Endothelial Growth Factor)</td>
<td>Platelets&lt;br&gt;Endothelium&lt;br&gt;Macrophages&lt;br&gt;Produced in oxygen depleted cells</td>
<td>Endothelium</td>
<td>- Angiogenesis&lt;br&gt;- Associated with types of cancers and metastasis</td>
</tr>
</tbody>
</table>