Can the Gingival Crevicular Fluid Transcriptome Predict Healing After Dental Trauma?

THESIS

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By

Christine Ann Vollmar

Graduate Program in Dentistry

The Ohio State University

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Master's Examination Committee:

Dennis McTigue, D.D.S., M.S., Advisor

Purnima Kumar B.D.S., M.S., PhD

Kumar Subramanian, D.D.S., M.S.D.

Ashok Kumar, D.D.S., M.S.
Abstract

Can the Gingival Crevicular Fluid Transcriptome Predict Healing After Dental Trauma?, Vollmar C, McTigue D, Kumar P, Subramanian K, Kumar A, (Nationwide Children’s Hospital and The Ohio State University, Columbus, OH)

Purpose: Determine if gingival crevicular fluid transcriptome is altered after dental trauma and if these changes persist over time.

Methods: Gingival crevicular fluid (GCF) samples were obtained from patients following permanent anterior tooth dental trauma who presented to Nationwide Children’s Hospital Emergency Department or Emergency Dental Clinic. Samples were obtained from the gingival sulcus using PerioPaper strip, placed in a sterile 10ml vial containing RNAlater, and stored at minus twenty degrees Fahrenheit. GCF sampling was repeated at routine follow-up encounters at 2-4 weeks, 6 weeks, 2 months, and 4-6 months when possible. Samples that were analyzed were divided into one of four categories of trauma: subluxation, uncomplicated crown fracture, extrusion, and avulsion. Four patients were selected from each group that demonstrated the most homogenous characteristics for sequencing and data analysis. RNA was isolated using epicenter baseline-ZERO DNase and Ribo-Zero Magnetic Kit. mRNA was enriched using ice-cold ethanol. cDNA conversion was performed using the Scriptseq kit from the stabilized mRNA. Pooled
cDNA libraries were clustered on the HiSeq and 250bp paired-end sequencing was performed. The filtered sequences were uploaded to the Kallisto program, which was used to align and quantify abundances of transcripts from the filtered sequences. Sequences were aligned to GRCh38 and estimated gene counts were obtained and uploaded in PANTHER (Protein Annotation Through Evolutionary Relationships) gene analysis platform for analysis. Statistical over-representation test with Bonferroni correction was carried out to obtain the difference in fold enrichment of genes and functions between the groups. Significantly different pathways were visualized using PANTHER mapping systems.

Results: At the initial encounter, the avulsion group shows the least number of gene transcript families present, whereas the uncomplicated crown fracture and extrusion groups had the most. By the final encounter, the avulsion group had the most transcript families expressed, while the crown fracture and extrusion groups showed the least. The subluxation group had an intermediate number of transcript families present immediately after dental trauma that slightly increased at the final visit. The quantity and quality of gene expression varied between groups at both the initial and final encounter. Looking at the PANTHER pathway maps, specific pathways were up regulated in different groups.

Conclusion: GCF Transcriptome displays changes in gene expression immediately after dental trauma representing a rapid response to injury at the level of the genome. The transcriptome remains elevated overtime in all groups, however, some types of trauma show more of a prolonged response while others return closer to baseline.
Dedication

This document is dedicated to my husband, Andrew Vollmar. Thank you for your love and support and for reminding me what is important in life.
Acknowledgments

I would like to thank my committee for the time, guidance and support in making this project a success. Without them, this project would not have been possible. I would also like to thank those in Dr. Purnima Kumar’s lab at The Ohio State University College of Dentistry; a special thanks to Sukirth Ganesan and Shareef Dabdoub for your efforts in data processing and analysis. Lastly, thank you to the pediatric dental residents at Nationwide Children’s Hospital who helped with sample collection. You guys are the best!
Vita

May 2005 ............................................. Louisville, OH, High School

May 2009 .............................................. B.A. Chemistry, Miami University, Oxford, OH

May 2013 .............................................. D.D.S., The Ohio State University College of Dentistry

2013 to present ...................................... Dental Resident, Division of Pediatric Dentistry, The Ohio State University

Fields of Study

Major Field: Dentistry
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Chapter 1: Introduction

Traumatic dental injuries are fairly common among school-aged children and adolescents accounting for about 5% of all injuries for which people seek treatment\(^1\). A 12-year review of the literature found that 25% of all school-aged children experience dental trauma, that the majority of dental injuries occur before the age of nineteen, and by adulthood 33% of adults have experienced trauma to the permanent dentition\(^2\). One study over a period of 6 years found a prevalence of 48.25% for dental injuries in 6000 patients with facial trauma\(^3\). Some researchers also claim the frequency of dental trauma and its sequelae are often underreported\(^1,3\).

The impact of dental trauma on the patient can have both short-term implications, such as pain, swelling, infection, and also long-term consequences such as ankylosis or potential tooth loss, extensive dental treatment needs, as well as financial, social, and psychological implications. Research in the area of dental trauma is important because timely diagnosis, proper treatment planning and follow-up care is crucial to ensure the best possible outcome for the patient\(^4-6\).

Traumatic dental injuries can vary in severity and cannot only affect the tooth structure itself, but also the surrounding periodontal structures such as the periodontal ligament (PDL) and the alveolar bone. In some injuries, the tooth can maintain its position within the socket and suffer only concussion or subluxation injury, for
example. However, when the tooth is displaced from the socket, such as in a luxation, extrusion or intrusion injury or is completely displaced removed the socket, as in an avulsion, a potentially more significant injury to the periodontal structures likely occurs. Many factors have been suggested that may have an impact on healing after dental trauma, including stage of root development, initial treatment performed, whether or not there was a crown fracture involved, and dry extra-alveolar storage period for avulsions. One source found that the two most significant predictors of pulpal necrosis after dental trauma are the stage of root development and the severity of the dental injury. However, very little is known about the responses of the periodontal ligament to traumatic injuries of the tooth, and also little is known about the effect of specifically non-displaced injuries on the periodontium. The impact from dental trauma can affect both pulpal and periodontal tissues, and maintaining their vitality is key to a favorable outcome.

The dental pulp contains nerves and blood vessels within the mineralized structures of the tooth, which maintains tooth vitality and helps to defend the tooth against pathogenic bacteria. After dental injury, if the vascular supply remains intact and the pulp maintains vitality, it should be able to defend itself from bacterial invasion and continue healing. However, when a dental injury occurs that compromises the vascular supply of the tooth, the pulp is unable to defend itself against bacterial invasion and is more likely suspected to become infected and undergo pulpal necrosis.

Bacterial infection of pulp tissue, if left untreated, can provide a persistent source of inflammation to the adjacent periodontal structures stimulating tissue breakdown, root resorption and increased osteoclastic activity. For this reason, it is imperative that pulp
tissue that becomes necrotic following dental injury be removed to prevent the process from perpetuating \textsuperscript{12,13}. This is consistent with the recommendation to expirate the pulp following dental avulsion of a closed apex permanent tooth within 7-10 days following the injury as the pulp would no longer be vital \textsuperscript{14}. If a necrotic pulp is not removed and inflammatory process continues, it can cause destruction of the tooth and surrounding periodontium with inflammatory or replacement resorption resulting \textsuperscript{11,13,15}.

The force from a dental injury is also transmitted to the root of the tooth and can cause cell death to the periodontal ligament cells in that area of impact. This denuded area of the root becomes chemotaxic to tissue healing cells, such as macrophages, osteoclasts, and other inflammatory components, which will try to repair the root and PDL cells at the site of injury \textsuperscript{12}. This infiltration of inflammatory cells and molecules is a normal part of the healing mechanism and is usually self-limiting, however, it can become pathogenic if homeostasis between pro-inflammatory and anti-inflammatory components is not achieved. If a balance is not achieved, osteoclasts and macrophages can dominate the infiltrate at the site of injury and remove damaged tissue at a quicker rate before allowing healing to take place \textsuperscript{16}. If the inflammatory process persists or is unregulated, increased osteoclastic activity will continue leading to resorption of the root \textsuperscript{12,13}. Bacterial infection of pulpal tissues and pulpal necrosis can provide continuous inflammatory stimulus, which further exacerbates resorption and osteoclastic activity within the periodontal tissues \textsuperscript{13,15}.

After dental trauma, the clinician must monitor the healing process of the tooth both clinically and radiographically and look for signs and symptoms which may
differentiate healing from necrosis, processes which often occur simultaneously and as part of a continuum \(^{10,11}\). Any evidence of arrested tooth development, peri-radicular periodontitis, external inflammatory root resorption, sinus tract formation, or clinical symptom of pain with percussion are all indicative of pulpal necrosis and would require endodontic therapy \(^ {11}\). However, with current diagnostic methods available, we are not able to absolutely determine if the disease process is arresting or progressing as we are only evaluating the tooth at a specific point in time.

The diagnosis of pulpal and periodontal pathology is sometimes inconsistent and signs of pathology often present after damage to periodontal tissues has already occurred. Current treatment for dental trauma is based on clinical and radiographic signs that often do not manifest until significant damage to the periodontal and pulpal tissues has begun. Furthermore, symptoms reported by the patient can occur inconsistently and often do not alert the patient to any underlying pathology until significant damage is done, sometimes even many years later when the inflammation has progressed. If proper treatment is not rendered at the appropriate time, inflammation and tissue damage can be exacerbated leading to a poorer prognosis for the tooth.

In order to provide the best clinical outcomes for patients with dental trauma, we need a more reliable and earlier predictor of healing. Investigating the presence of inflammatory responses at the site of injury may give us some insight into healing following dental trauma. If we could detect increased inflammatory processes at the molecular level before tissue destruction has occurred, and intervene as needed, we may be able to prevent resorption and damage to the root well before radiographic or clinical
signs have manifested.

Gingival crevicular fluid (GCF) is a biological substance secreted within the gingival sulcus of a tooth, and it can exist as either a serum transudate or perhaps more commonly in a disease state as an inflammatory exudate. It contains various cellular and biochemical molecules important for immune function, cell growth and cell repair. It also contains enzymes and tissue breakdown products, which can reflect the physiological status of periodontal ligament during the healing process. Gingival crevicular fluid has been used in numerous studies of periodontal disease and orthodontic root resorption. GCF is readily available within the periodontal sulcus and its collection is simple and non-invasive to the patient. The gingival sulcus is intimately associated with the periodontal ligament and periapical tissues, and we would suspect that its fluid composition following traumatic dental injury would contain abundant inflammatory cells and biochemical molecules. This inflammatory response could provide valuable insight into healing after dental trauma.

Previous studies of the GCF after dental trauma and the differences in cytokine profiles have been conducted at Nationwide Children’s Hospital in the past several years. These studies have shown that the presence of certain pro-inflammatory cytokines have differed between avulsed teeth that eventually ankylose and those avulsed teeth which do not undergo ankylosis. Another study looking at displaced dental injuries versus non-displaced dental injuries and their controls and have shown significant differences in cytokine profiles as well. Therefore, since we know that there are differences at the level of the cytokine in response to dental trauma, this study is
interested in analyzing the differences at the transcriptome level, which are responsible for the expression of cytokine differences observed.

Previous studies in microbiology, medicine and dentistry have discovered associations between the presence and function of certain cytokines and various disease or inflammatory processes. Cytokines and chemokines are proteins secreted by the body intimately involved in the activation of an immune response or inflammatory reaction and participate in healing after an injury 26. Cytokines are critical for healing, however, healing is a multi-factorial process involving several different pathways. At the cellular level, the production and differentiation of various cytokines as well as their cascade of responses depends on the immune cells present at the site and their interaction with that cytokine. 26 From a broader scope, however, the production of cytokines, and in fact, all cell functions are ultimately controlled by the genetic message within the cells and how that message, or transcriptome, is expressed in response to a stimulus.

All cells within the human body contain DNA, which is the double-stranded molecule that contains the instructions used to build and maintain cells. These instructions are coded as the sequence of four chemical base pairs, which are organized within the gene. The instructions are transcribed into mRNA, which is used by the cell to perform all cellular functions, such as cell growth and repair, protein synthesis, and molecular signaling. This message is called a “transcript” and the collection of gene transcripts is called the “transcriptome” 27. Depending on the needs of the cell at the time or in response to a stimulus, the transcriptome can vary to provide the necessary messages for cell function and response.
Nearly every cell in a person’s body contains the same DNA, yet not all cells perform the same functions and each cell can also show different patterns of gene expression in response to a stimulus. By analyzing the transcriptome, researchers can determine where a gene is turned on or off and the amount of gene expression in a certain cell at that point in time. The activity of certain genes can be up-regulated or down-regulated depending on the needs of the cell in response to a stimulus. The traditional paradigm of the host response to injury includes an early up-regulation of the pro-inflammatory response followed by a compensatory anti-inflammatory response and suppression of the adaptive immune response. However, some studies have proposed a new model that involves rapid up-regulation of both pro-inflammatory and anti-inflammatory gene expression simultaneously with suppression of the adaptive immune response 28.

When analyzing the response of a tooth to traumatic injury, the transcriptome of the gingival crevicular fluid could serve as a reflection of the body’s inflammatory response displaying altered gene expression as the tissues attempt to repair. In a similar way, previous studies have analyzed the transcriptome of biological secretions in connection with normal development as well as disease states. One study has used the amniotic fluid transcriptome and gene expression to analyze the normal development of the fetus in utero. They have also identified the presence of unique fetal transcriptomes common to certain prenatal disease processes, such as Down Syndrome and Turner Syndrome. These fetal transcriptomes unique to certain disease are then demonstrated as a specific disruption in a molecular pathway leading to the disease manifestation.
observed 29.

The human transcriptome has already been used to study the immune response to traumatic injury in medicine. A multi-center clinical study analyzed the circulating leukocyte transcriptome following severe trauma and burn injuries and found that within the first 28 days after injury more than 80% of the leukocyte transcriptome expression was altered with the greatest changes in leukocyte gene expression occurring within the first twelve hours. Of the genes whose expression was altered, those that increased the most were involved in the innate immune response and inflammatory reaction. Surprisingly, when comparing outcomes of complicated recovery with uncomplicated recovery, they found that the quality of gene expression patterns were highly comparable and reproducible; however, those with a complicated recovery differed in the magnitude of the initial response and had a prolonged alteration of gene expression before returning to baseline 28.

Human transcriptomics is a more open-ended approach to the cell’s response after injury than has ever been used before to study dental trauma. This study will be analyzing gene expression as a “snap-shot” in time, not only during the initial response to injury but also looking at the development of complications and delayed healing following traumatic dental injury to determine how our cells are responding and if the transcriptome can be used to predict healing. Because not all types of dental trauma heal in the same way, we hypothesize that this genetic response may influence how a tooth heals after injury. With information about the gingival crevicular fluid transcriptome of a traumatized tooth compared to a non-traumatized tooth we may be able to more reliably
predict healing outcomes after dental trauma. With this information, we may be able to alter the body’s immune response to the injury to prevent tissue destruction before it progresses, thus resulting in better clinical outcomes.

Hypothesis: The GCF transcriptome will be altered immediately following dental injury and these changes overtime will correlate with the healing process.
Chapter 2: Methods

A. Sample Collection

Research protocol and consent forms were reviewed by the Institutional Review Board (IRB) at Nationwide Children’s Hospital. Patients from ages 8-18 years old from both Nationwide Children’s Hospital Emergency Department and Nationwide Children’s Hospital Dental Clinic who experienced dental trauma to their permanent anterior teeth were approached to be included the study.

Any patient with any maxillary or mandibular permanent anterior tooth trauma was eligible to participate; however, subjects were initially excluded from the study if the trauma occurred more than 24 hours prior to their initial presentation, if they had any previous trauma to the tooth, or if there was any active infection in the area of the dental trauma. Only patients who were ASA 1 or ASA 2 were included in the study; therefore, patients were excluded from the study if they had any systemic disease such as diabetes, immunosuppressive therapy or hematologic disorders, or if they were smokers. The research objectives and study procedures were explained to the patient and legal guardian, and those who chose to participate signed an informed consent form approved by the Nationwide Children’s Hospital Institutional Review Board (IRB). Patients over the age of nine were asked to sign an assent form also approved by the IRB.

Participation in the study was completely voluntary and did not affect the
initial treatment rendered or any follow-up procedures. Patients could still receive follow-up dental trauma treatment at Nationwide Children’s Dental Clinic if they chose not to participate. All treatment was rendered according to the American Academy of Pediatric Dentistry Guidelines for the Management of Traumatic Dental Injuries and the International Association for Dental Traumatology Guidelines \(^{14,30}\). All treatment was performed by dental residents at Nationwide Children’s Hospital in Columbus, Ohio and overseen by three dental faculty members.

After emergency treatment was rendered, the traumatized clinical crown(s) were individually wiped with gauze to remove supragingival dental plaque, saliva or blood, so as not to contaminate the sample. The teeth were isolated with cotton rolls and gingival crevicular fluid (GCF) samples were taken individually with PerioPaper strips (Oraflow) in six locations around the sulcus of the tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, mesiolingual). The strips were removed from the sulcus after approximately twenty seconds and were placed in a sterile 10ml vial containing RNAlater (Ambion, The RNA Company). RNAlater solution was used to ensure stability of the samples until properly stored. The vials were sealed and stored in a freezer at minus twenty degrees Fahrenheit. This procedure was repeated for each traumatized tooth as well as one permanent control tooth at each follow-up encounter. The control tooth was selected as an adjacent non-traumatized permanent anterior tooth within the same arch. If not possible, due to pattern of dental eruption at the time of injury, a control tooth from the contralateral arch was used.

Samples were collected at the initial encounter and at follow-up appointments
after approximately 2-4 weeks, 6 weeks, 2 months, and 4-6 months after the initial trauma. Some patients required additional follow-up appointments after this time frame and samples were also taken until no further follow-up was required or study sample collection ended.

At the initial encounter, demographic information was recorded for each subject including age, date of birth, race, and gender. Data regarding the dental trauma was also recorded including tooth number(s) involved and control tooth selected, etiology of trauma, type of trauma sustained to each tooth, root stage development of the tooth or teeth, time elapsed from initial trauma to initial encounter, procedures performed at initial encounter and follow-up interval recommended.

At each follow-up visit, radiographs were taken and pulp vitality testing was performed. History of symptoms were obtained from the patient including any pain, soreness, cold sensitivity, pain to biting or chewing, mobility, swelling. Clinical findings at follow-up visits were recorded including, response to cold testing, electric pulp test (EPT), tooth mobility, percussion sensitivity, and tooth discoloration. Other information recorded was the time elapsed between follow-up encounters, follow-up procedures performed at each visit, including if any pulp therapy was required. Radiographic findings were also recorded including periapical pathology, widened PDL, integrity of lamina dura, external root resorption, internal root resorption, and replacement resorption.

In this study we were interested in comparing the healing response between those injuries that caused tooth displacement versus those injuries that caused no displacement and if there was any difference in the transcriptome at the initial visit compared to the
final visit. Those teeth with no displacement that we were interested in studying were grouped into two categories: concussion/subluxation and concussion/subluxation with uncomplicated crown fracture. Teeth with displacement injuries that we were interested in studying were grouped into two categories: avulsion and extrusion.

B. Lab Methods and Sequence Analysis

Gingival crevicular fluid strips stored in RNAlater were removed from storage at minus twenty degrees Fahrenheit. Total RNA then underwent rigorous DNase treatment using epicentre baseline-ZERO DNase. rRNA removal was accomplished using the bead-based approach Ribo-Zero Magnetic Kit (Illumina). mRNA was enriched with ice-cold ethanol and stabilized with 20 ul of RNA stable. Riboguard RNase inhibitor was used at every step possible to prevent degradation of RNA in the samples. Because mRNA is not stable, the mRNA had to be converted to cDNA for sequencing. cDNA conversion followed by library preparation was performed using the Scriptseq kit from the stabilized mRNA. Pooled cDNA libraries were clustered on the HiSeq (High Through-put Sequencing), and 250bp paired-end sequencing was carried out. The Illumina base-calling pipeline was used to process the raw fluorescence images and call sequences. Raw reads with >10% unknown nucleotides or with >50% low quality nucleotides (quality value <20) were discarded.

Our collaborations with the Ohio Supercomputing Center have allowed us to establish a high-throughput analysis pipeline for efficient large-scale sequence data processing. We are able to harness the incredible power of two parallel processor clusters (GLENN and OAKLEY), allowing us to generate data from multiple samples within a
few days. To begin with, bacterial sequences were filtered out. We used Kallisto, a novel program to align and quantify abundances of transcripts from the filtered sequences. Filtered sequences were uploaded to Kallisto, aligned to GRCh38 (human reference genome), and estimated gene counts were obtained. Gene counts output from Kallisto was then uploaded to PANTHER (Protein Annotation Through Evolutionary Relationship) gene analysis platform (http://www.pantherdb.org/) for further analysis. PANTHER platform provides several methods to classify genes based on their functions, and group those functions into different modules. Statistical over-representation test with Bonferroni correction was carried out to analyze the differences in fold enrichment of genes and functions between the groups. Global gene expression was annotated with GO (Gene Ontology) and significantly different pathways were visualized using the PANTHER mapping system. The Gene Ontology (GO) project is a collaborative effort to address the need for consistent descriptions of gene products across multiple collaborating databases.
Chapter 3: Results

A total of 118 patients with 191 traumatized teeth were initially included in the study. From this sample, we selected four types of trauma that we were interested in analyzing: avulsion, extrusion, concussion/subluxation, and concussion/subluxation with uncomplicated crown fracture. In each of the four categories, four injured teeth that had the most homogenous characteristics and consistent follow-up were selected for analysis. Type of trauma, duration between initial and final sample collection, and total number of encounters during duration of study and patient demographic information were gathered and are shown Table 1.

<table>
<thead>
<tr>
<th>Type of Injury</th>
<th>Duration</th>
<th># visits</th>
<th>Age</th>
<th>Race</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avulsion</td>
<td>1 mo</td>
<td>4</td>
<td>14</td>
<td>Caucasian</td>
<td>Male</td>
</tr>
<tr>
<td>Avulsion</td>
<td>2 mo</td>
<td>4</td>
<td>8</td>
<td>Caucasian</td>
<td>Male</td>
</tr>
<tr>
<td>Avulsion</td>
<td>7 mo</td>
<td>5</td>
<td>11</td>
<td>Caucasian</td>
<td>Female</td>
</tr>
<tr>
<td>Avulsion</td>
<td>7 mo</td>
<td>5</td>
<td>11</td>
<td>Caucasian</td>
<td>Female</td>
</tr>
<tr>
<td>Extrusion</td>
<td>2 mo</td>
<td>4</td>
<td>13</td>
<td>Caucasian</td>
<td>Female</td>
</tr>
<tr>
<td>Extrusion</td>
<td>2 mo</td>
<td>3</td>
<td>15</td>
<td>Caucasian</td>
<td>Male</td>
</tr>
<tr>
<td>Extrusion</td>
<td>3 mo</td>
<td>3</td>
<td>16</td>
<td>Hispanic</td>
<td>Male</td>
</tr>
<tr>
<td>Extrusion</td>
<td>5 mo</td>
<td>4</td>
<td>10</td>
<td>Caucasian</td>
<td>Male</td>
</tr>
<tr>
<td>Concussion/Subluxation Only</td>
<td>2 mo</td>
<td>4</td>
<td>11</td>
<td>Caucasian</td>
<td>Male</td>
</tr>
<tr>
<td>Concussion/Subluxation Only</td>
<td>2 mo</td>
<td>3</td>
<td>12</td>
<td>Somali</td>
<td>Male</td>
</tr>
<tr>
<td>Concussion/Subluxation Only</td>
<td>2 mo</td>
<td>3</td>
<td>11</td>
<td>Caucasian</td>
<td>Female</td>
</tr>
<tr>
<td>Concussion/Subluxation Only</td>
<td>4.5 mo</td>
<td>4</td>
<td>8</td>
<td>Caucasian</td>
<td>Female</td>
</tr>
<tr>
<td>Uncomplicated Crown Fracture</td>
<td>2 mo</td>
<td>3</td>
<td>8</td>
<td>Caucasian</td>
<td>Female</td>
</tr>
<tr>
<td>Uncomplicated Crown Fracture</td>
<td>3.5 mo</td>
<td>3</td>
<td>9</td>
<td>Somali</td>
<td>Male</td>
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<tr>
<td>Uncomplicated Crown Fracture</td>
<td>4 mo</td>
<td>3</td>
<td>11</td>
<td>Unknown</td>
<td>Female</td>
</tr>
</tbody>
</table>

Table 1: Sample characteristics and demographic data
The samples were classified into one of four categories: avulsion, extrusion, concussion/subluxation only (referred to as “subluxation” from here throughout), and concussion/subluxation with uncomplicated crown fracture (referred to as “crown fracture” from here throughout). Each type of trauma was analyzed as a group which had an initial and final average which will be referred to as follows: avulsion first (AF), avulsion last (AL), extrusion first (EF), extrusion last (EL), subluxation first (SF), subluxation last (SL), crown fracture first (CFF), crown fracture last (CFL). When the transcriptome of the GCF from our samples was compared to the human reference genome (GRCh38) for baseline, only those groups with alterations of statistical significance (p<0.05) were considered.

The total numbers of functional gene families whose expression varied with statistical significance compared to the human reference genome for each group of trauma at the initial visit and final visit are shown in Figure 1.

Figure 1: Number of gene families differing from GRCh38
Of all the initial visits immediately after trauma, the avulsion group had the least number of significantly different gene family transcripts that were expressed compared to other types of trauma; however, the avulsion group had the greatest total number of gene families by the last visit (AF = 28, AL = 114). Conversely, the crown fracture group had the greatest total number of gene family transcripts at the first visit but then had the fewest number at the last visit (CFF = 255, CFL = 16). The extrusion group also showed a decrease in the number of gene families expressed from the first to the last visit, however not as wide of a range as the uncomplicated fracture group (EF = 160, EL = 31). Finally, the subluxation group showed the least amount of change from the first to the last with a small increase in the number of gene families (SF = 58, SL = 72).

We see an interesting trend in overall gene expression following dental trauma. Compared to the GRCh38, all four types of dental trauma showed an increased expression of multiple gene family transcriptomes immediately after trauma (Figure 2).
Figure 2: GCF Transcriptome immediately following dental trauma

It is also important to note that by the last visit, all types of dental trauma still displayed enrichment of the transcriptome and had not yet returned to the baseline of GRCh38 (Figure 3).
Figure 3: GCF Transcriptome at final encounter

Of the gene families present, we selected certain categories specific to healing and repair to look at in more detail. Looking at each group’s transcriptome families immediately after injury, the GCF samples for all types of trauma display the common theme that gene families relating to sensory perception, seen in purple in Figures 2 and 3, were all down-regulated, thus experienced a decrease in sensory stimuli at the time period immediately after trauma. All of these values were only slightly varied from GRCh38
with -0.2 to -0.6 fold decrease, yet their significance was very strong in most cases (p <0.0001). Avulsion injuries had the fewest number of gene families relating to sensory perception compared to GRCh38, thus experienced the greatest loss of sensation. It is also interesting to note that sensory perception families were the only group of gene families that had a negative fold increase, all others had a positive fold increase in transcription compared to GRCh38. Also, all types of trauma had a remaining down regulation of sensory gene expression at the last visit except for the CFL group, which showed that there was no significant difference in sensory perception gene transcription compared to GRCh38 baseline and healing of sensory perception had occurred.

We also observed some common trends in other gene families at the first visit. After initial trauma, all of the groups of injuries had increases in gene families with metabolic and biosynthetic functions, shown in blue in Figures 2 and 3. Most of these fold enrichment values ranged from 1.16 to 1.40, however, the CFF group had some genes with up to 3 fold increases compared to GRCh38. Except for the avulsion group, all types of trauma had evidence of increased transcription of genes coding for immune system responses and inflammation immediately after trauma with fold enrichment ranging from 1.37 to 1.9. Due to the nature of avulsion injuries, we would expect less vital cells to render an immune system response thus less transcription at this time.

Other gene families present after initial trauma were more unique to only one or two types of trauma. For example, gene families relating to antigen processing via MHC class II, which is seen in CD4+ T-cell immunity and shown in green in Figures 2 and 3, were only seen in extrusion and subluxation injuries. It is also important to note that this
was a greater than 5 fold increase compared to GRCh38. These same two types of trauma, extrusions and subluxations, were also the only two types of trauma that displayed fold enrichment in actin cytoskeleton filament processes, seen in red in Figures 2 and 3. Lastly, only the crown fracture group immediately after injury displayed alterations in lipoprotein oxidation gene families, which are shown in pink in Figures 2 and 3. This gene family had an increased expression of a magnitude greater than 5 fold increases representing a high metabolic demand during healing. No other type of trauma displayed alteration in the lipoprotein oxidation gene family.

Immediately post-trauma, each type of injury also displayed a different characteristic profile of gene transcription. After avulsion, the GCF transcriptome showed the least number of gene expression families compared to the other three groups of trauma. Uncomplicated crown fracture samples showed a higher fold increase in metabolic and biosynthetic systems than avulsed teeth and immune responses, shown in orange in Figure 2 and 3, were also up regulated where they were not present in avulsion injuries. The subluxation group had less initial inflammatory response than the extrusion group, and both still had less inflammation than uncomplicated fracture groups.

At the last visit, all groups demonstrated persistent decreased sensory perception except for the uncomplicated concussion group, which seemed to return to normal. At the last visit, increased gene transcription in immune response and inflammation, seen in orange in Figure 3, were only seen in the avulsion group, which is interesting because this was the only type of trauma that did not have increased immune and inflammatory responses immediately after trauma. This would be consistent with the fact that avulsed
teeth and PDL cells become non-vital immediately after injury but later are trying to heal evidenced by increased transcription at the last visit. Numerous metabolic and biosynthetic gene families are still up regulated in the AL and SL, whereas they are present but not as pronounced in the extrusion and uncomplicated fracture groups.

Antigen-processing genes via MHC class II processes, seen in green in Figure 2, were only present with initial extrusions and subluxations (EF and SF). These gene processes were not present immediately after trauma with avulsion or crown fractures. It is interesting to note that compared to the initial visits, antigen-processing genes were no longer seen in the extrusion and subluxation groups, rather they were only seen with avulsion and crown fracture groups, and they no longer involved MHC class II but rather the MHC class I at the last visits. The major difference between MHC (Major Histocompatibility Complex) Proteins I and II is that MHC I proteins interact mainly with cytotoxic T cells and are involved with endogenous antigens whereas MHC II proteins interact mainly with helper T cells and are involved with exogenous antigens.

Several different pathway maps can be displayed showing differences in gene expression after dental trauma. We chose two difference pathways relating to inflammation after trauma to highlight increased activity in the inflammation mediated by chemokine and cytokine signaling pathway and toll-like receptor signaling pathway, displayed in Figure 4 and Figure 5 respectively.
Figure 4: Inflammation mediated by chemokine and cytokine signaling pathway

Figure 5: Toll-like receptor signaling pathway
Figure 4 shows increased cytokine and chemokine signaling specific to each type of trauma. The areas in green correspond to extrusion injuries, which demonstrate an increase in pro-inflammatory genes and pro-inflammatory anti-apoptosis, whereas the red areas corresponding to subluxation injuries show increased expression of plasma membrane phospholipid, arachidonic acid, prostaglandin and leukotrienes. Avulsion injuries, which are shown in blue, have increased expression in IP3 receptors, which are important for signal transduction.

Figure 5, which displays a pathway map for toll-like receptors highlighting areas in yellow that have increased activity in 2 or more groups. Toll-like receptors are involved in the innate immune system, also known as the non-specific immune system, and are up regulated in the presence of bacteria. It can be seen that in response to dental trauma, an increased gene expression in toll-like receptors on the cell surface correlates to an increase in gene transcription seen by the bright yellow within the nucleus.
Chapter 4: Discussion

Comparing the transcriptome of the GCF immediately after trauma and at the final visit, we can appreciate the changes in gene transcription overtime and correlate them with healing outcomes. Overall, a decrease in transcriptome expression can be appreciated in extrusion injuries and crown fractures, which would indicate the healing process has continued and brought the transcriptome profile closer to GRCh38 baseline. However, there is an increase in gene transcription in the avulsion and subluxation groups overtime.

With the avulsion injuries, which clinically have delayed healing and often result in ankylosis, we may have expected this prolonged increase in transcript profile. The increase in transcription at the final visit correlates with a prolonged inflammatory response and potential for ankylosis. This finding of a delayed response correlating with a more complicated recovery is consistent with the findings of the previous study by Xiao et al. However, this same study also found that a more complicated recovery resulted when transcription was increased in magnitude after the initial injury. We do not see this initial increase in transcription immediately after avulsion due to the loss of vitality of pulpal and PDL cells. Because transcriptomics is only looking at a “snap-shot” in time, it is possible that we are missing the large magnitude increase in transcription for avulsion injuries that Xiao et al. is suggesting. Perhaps if GCF samples were taken at a
more frequent interval, we may be able to observe this rapid increase at another time point rather than immediately after injury for the avulsion group.

Comparing the two types of displaced injuries, avulsions and extrusions, we see that the extrusions have a more pronounced increase in transcription after the initial injury compared to the avulsion group. This could be attributed to the fact that in extrusion injuries, the PDL cells are able to maintain vitality as they are still, for the most part, submerged within the alveolar socket. In contrast, the PDL cells after avulsion quickly lose vitality due to desiccation from being out of the socket. It could also be suspected that the vitality of the PDL cells in the extrusion group are critical during the healing process and actually help the extrusion injuries heal more rapidly, whereas the avulsion group with most likely non-vital PDL cells have a more prolonged inflammatory response during healing.

Delayed healing and prolonged alterations in gene transcription may not have been expected with the subluxation group, however. One possible explanation for this observation could be that the observation period for these types of injury is often of shorter duration as clinically and radiographically there may be no signs of delayed healing. Furthermore, in our subluxation group, the final visit for three out of four of the patients was only after two months, so perhaps there was not enough time to see healing. Also, if there were no signs or symptoms of pulpal necrosis, a pulpectomy would not have been warranted, as it would have been in an avulsion or an extrusion of a closed apex tooth, for example. For this reason, we may be seeing a prolonged healing response due to the fact that the pulpal inflammation, whether reversible or irreversible, may be
persisting as well. It could also be argued that an increase in transcription after injury is necessary for healing and that perhaps a greater response correlates to more rapid healing. In this way, it could be assumed that because the subluxation group does not have as large of an initial response, compared to the crown fracture group for example, perhaps it was not able to heal as quickly.

Interpreting the results of the crown fracture group also brings up some interesting discussion points. In a clinical setting, some may typically observe more pulpal necrosis and inflammation with a crown fracture than with a subluxation, for example. In our study, based on the amount of transcription occurring at the final visits for these groups we observe that the crown fractures appear to have experienced more healing than the subluxations; therefore, with our results we would anticipate less complications with a crown fracture and more potential for complications with subluxations. This is contrary to what is often observed clinically and could be explained due to the fact that only uncomplicated crown fractures with no pulp exposure or pulpal blushing were included for data analysis. In addition, all of the patients with crown fractures that were included were considered “complete” with trauma re-evaluation whereas in the other groups, only one of four patients were considered “complete”.

Gene expression after healing can be pro-inflammatory and anti-inflammatory and both play an important role in normal healing. After looking at the trends in our results, it could be hypothesized that an initial increase in gene transcription is necessary to aid in the inflammatory response and may help facilitate cell repair and healing at a more rapid rate. Therefore, the increased transcript activity after injury with the extrusion
and crown fracture groups may actually help with healing at a faster rate and thus we see less transcription at the last visit for these two groups. In the same way, we do not see as significant of a response immediately after avulsion or subluxation and these same groups have more altered transcription at the last visit.

There were several limitations to this study including that we were unable to control for various patient factors. For example, we were not able to control for the patient’s baseline oral hygiene, which could affect baseline gingival inflammation present within the GCF samples. We also did not record whether or not that patient had taken any anti-inflammatory medications, which could affect the transcriptome and the healing response. Four of the patients in our study had a history of asthma and were taking either beta-2 agonist and/or a steroid inhaler, which may have affected their baseline inflammatory response. Patient compliance was also an issue with our study and there was a wide variation in duration between follow-up encounters. There were also a number of different providers who obtained samples as part of the study, which could not be avoided due to our clinical setting. Lastly, we limited our pilot study to include only four patients with each type of trauma due to the high costs to sequence the genome; therefore, our sample size was limited.

This study used transcriptomics to look at the response after dental trauma from a global perspective and we observed that indeed the GCF transcriptome is altered after injury and that it varies between groups based on the type of trauma that occurred. In this pilot study, we limited our analysis to a few samples and only initial and final visits for a broader interpretation. Using the method of transcriptomics in future studies, one might
be able to compare clinical outcomes of each specific patient with their gene transcription response including several different time points after injury to gain a better understanding of the healing process. Since transcriptomics is a “snap-shot” in time, it would be interesting to see if the transcription profiles we observed at the beginning and end were consistent throughout if more time intervals were included. Also, since we observed increased expression in arachidonic acid pathways in subluxation injuries and increased pro-inflammatory genes in the extrusion injuries, we would suspect that taking certain medications might have an effect on healing after dental trauma. A future study could analyze the transcriptome after providing NSAIDs versus cox-2 inhibitors versus no medication to different groups to see if these interventions have any effect on gene expression and healing.
Chapter 5: Conclusions

We are able to conclude that the GCF transcriptome does display changes in gene expression immediately after traumatic dental injuries compared to our reference genome. These alterations represent a rapid response to injury at the molecular level. We also know that the initial response in transcription varies in quality and quantity depending on the type of trauma. These alterations in the transcriptome persist over-time during the healing process with some types of trauma displaying a more prolonged response while others return closer to baseline.
References