INTRODUCTION

Inflammation of the dental pulp (pulpitis) can be a progressive and devastating pain experience characterized by spontaneous or provoked pain, hyperalgesia, allodynia and difficulty in achieving adequate local anesthesia. The activation of dental pulp or periapical nociceptors during endodontic inflammation elicits a pain response that contributes to approximately 90% of dental emergency visits in both private dental clinics and in hospitals. The economic implication of these emergency visits is reported to cost almost US$1 billion per year. However, despite the cost and the prevalence of endodontic disease and the great discomfort associated with it, the fundamental molecular aspects of its pathogenesis are still not fully understood. The current literature on pulp immune response to microbial infection continues to expand but very little is known on the regulatory mechanisms behind pulp disease.

Cells comprising the human dental pulp trigger immune responses to a complex array of microorganisms that invade dental tissues. These immunocompetent cells also form mechanical barriers (that is, odontoblasts), detect and transmit sensations (nerve fibers) or differentiate (that is, dental pulp stem cells) to limit infection, signal injury and promote repair, respectively. These cascades of events resulting from dental pulp stimulation by microorganisms result in the release of a plethora of immune mediators that trigger pulpal or odontogenic pain, inflammation or in advanced stages, pulp necrosis. In addition, several studies have suggested that pulpal events can be reflected in the gingival crevicular fluid through measurable levels of protein markers that correlated with pulpal symptoms. This shows that the dental pulp is not an isolated environment but rather a vital, reactive tissue that communicates with the outside environment.

Several studies have identified the biological differences between healthy and inflamed dental pulp. Cytokines, cell surface receptors and other protein markers are shown to be either highly increased or decreased in inflamed dental pulp. A limited number of studies have examined gene expression in inflamed human pulps. These studies, however, did not explore the differences in gene expression between normal and inflamed dental pulp and the clinical presentation of donor patients (that is, pain and swelling), which may provide biologic explanation on the variability of clinical signs and symptoms of pulp inflammation that confound diagnosis.

Although the clinical value of a molecular diagnostic marker may at first appear limited in scope, the emerging correlation of gene expression with clinical signs and symptoms will enhance our understanding on the development of pulp inflammation—this will add not only to the knowledge base but it will also provide a biological basis for the varying clinical presentations of pulpitis. Prior studies that focused on histological findings have shown a wide variation—from poor to strong—in correlating clinical signs and symptoms with histological findings. In this study, data from the full genome scan will be utilized to determine if an association exists between gene expression and clinical presentation (that is, pain) of pulpitis patients.

RESULTS AND DISCUSSION

Normal and pulpitis samples exhibited differentially expressed genes

The SAM software generated gene set enrichment analysis (GSEA) data, which showed a significantly higher expression of various gene sets that are associated with immune response activation, maintaining cellular function and cell-to-cell interaction, among
others (Figure 1) in pulpitis samples. This underscores the utility of both the subjective (patient-derived history) and objective (endodontist-performed testing) diagnostic techniques that clinically delineate a normal from an inflamed dental pulp. Furthermore, the results above re-establish the immunocompetency of the dental pulp that has been shown to carry Toll-like receptor (TLR) \(-2/4+\) cells.\(^{7,28}\)

Differences in gene expression between mild and moderate to severe pain

Among the patients diagnosed with irreversible pulpitis (IP), 8 patients reported experiencing zero to mild pain \((\leq 30 \text{ mm on visual analog scale (VAS)})\) and 12 patient reported moderate to severe pain. The mean VAS scores for patients who reported zero to mild pain and moderate to severe pain was 6.63 (s.d. = 9.29) and 81.93 (s.d. = 11.21), respectively \((P < 0.0001)\). There were differentially expressed genes between the two groups (Figure 2; Supplementary Figures S1 and S2). Mild pain samples showed a significantly higher expression of genes involved in adaptive immune system, cytokine to cytokine interaction and cytokine signaling, among others. However, when looking at specific genes, several of them that have key roles in inflammation are significantly underexpressed, unchanged or overexpressed in asymptomatic or mild pain patients compared with those with moderate to severe pain (Table 1).

Relief from pain is a very important part in the practice of endodontics. Patients often judge the success of treatment and the efficiency of the dentist based on their pain experience. The decision whether to perform root canal therapy to relieve pain or manage infection relies heavily on clinical diagnostic tests, which dichotomize the pulpal diagnosis to reversible and IP. To arrive at a diagnosis, dentists depend mainly on pain history and responses to sensibility tests (namely thermal tests and electric pulp testing), which have been used for decades; but whether the responses to these clinical tests correlate well with pulpal histopathology or not remains unclear.\(^{25–27,29–31}\)

This study was aimed to identify the differences in gene expression between normal pulps (NPs) and pulpitis samples and between mild and moderate to severe pulpitis pain, based on the diagnostic guidelines set by the American Association of Endodontists (AAE) and on the patient-reported pain experience at the time of endodontic treatment, respectively. GSEA was used for the analysis of the thousands of genes included in the microarray screening.\(^{32,33}\) The Broad Institute defines GSEA as a computational method that determines whether an \textit{a priori} defined set of genes shows statistically significant, concordant differences between two biological states.

Several applications are envisioned for the data that were gathered in this study. First, a biological basis at the molecular level can now be correlated not only with the current diagnostic testing procedures but also with the diagnosis of IP itself. Molecular alterations precede histopathological changes and in the case of the dental pulp, there can be a wide array of histological presentations depending on the timing of pulp extirpation; thus, there are conflicting reports concerning the clinical presentation and actual histopathology of the pulp. Studies investigating inflammation at the molecular level may therefore provide a more accurate representation of the inflammatory changes in the dental pulp.

Next, the results may be used to cluster genes that are overexpressed or underexpressed in inflamed pulps and to use this information as biological basis for future studies on acute pain and on oral biology, especially those that focus on odontogenic pain and regeneration as these studies require a broad understanding of molecular changes that dictate clinical and biological outcomes. The difference in gene expression between normal and inflamed dental pulps (Figure 1) may have been the expected result considering that cytokines, chemokines and their receptors

![Figure 1](https://example.com/figure1.png)

**Figure 1.** GSEA results between pulpitis and normal samples. Each bar represents the functional categories and the number of significantly regulated genes between pulpitis and normal groups \((q < 0.05)\).

![Figure 2](https://example.com/figure2.png)

**Figure 2.** GSEA results between groups that reported none to mild pain and those that reported moderate to severe pain on VAS. Each bar represents the functional categories and the number of significantly regulated genes between none to mild and moderate to severe pain groups \((q < 0.05)\).
are known to be overexpressed in inflammatory conditions; however, this study is still an important addition to our current understanding of pulp biology as it provides a broad picture of the molecular changes occurring in the dental pulp during the very moment that the patient is seeking endodontic treatment on the dental chair.

Another remarkable finding in the study is the overexpressed genes related to inflammation and immune response in pulps from patients who reported zero to mild pain (≤30 mm on VAS) during their endodontic appointment compared with those who had moderate to severe pain (Figure 2). Moreover, looking at individual genes separately from the a priori gene sets in the GSEA analysis during the pathological stimuli. TLRs are key molecules in the response to pathological stimuli. They recognize viral and microbial structures, as well as self molecules (such as single-stranded RNAs) that may accumulate in non-physiologic amounts or sites. 39,40 They recognize viral and microbial structures, as well as self molecules (such as single-stranded RNAs) that may accumulate in non-physiologic amounts or sites.

Pathogen-associated molecular pattern receptors, including TLRs, are key molecules in the response to pathological stimuli. Pulpitis is a disease that is mainly due to microbial insults. TLRs are expressed by both immune and non-immune cells in the pulp including neurons, fibroblasts, endothelial cells, epithelial cells and others. They recognize viral and microbial structures, as well as self molecules (such as single-stranded RNAs) that may accumulate in non-physiologic amounts or sites. 39,40 TLR ligands stimulate the production of pro-pain molecules and, in this way, may contribute to the pathogenesis of pain. 41,42 In this study, the expression of several TLRs (1, 2, 4, 6, 8 and 9) were upregulated and TLR3 was downregulated in inflamed pulps as compared with NPs. Our data also show that expression of TLR8 was higher in pulpitis patients experiencing severe pain as compared with those who reported no pain or mild pain. This is of particular interest given the recent finding that murine TLR7 (which is phylogenetically and structurally related to human TLR8) interacts with TRPA1 and that specific miRNAs activate nociceptors via TLR7 and TRPA1. 43–45

Arriving at a reliable diagnosis that matches the histopathology of the dental pulp, especially during a pain episode is arguably the most challenging part of endodontics. The dental pulp is a dynamic tissue that is capable of responding to insults and of healing so that diagnosis may change over time. 46 Furthermore, individual variations in immunologic and pain response further complicate not only the clinical but also the biological aspect of endodontic diagnosis. This may be the one of the main reasons for the observational differences among studies correlating clinical and histopathological diagnosis as pointed out in the earlier part of the article. Understanding the molecular changes in pulps may help develop better diagnostic tools. For example, molecular markers collected from dentinal fluids may be used to determine the pulp status. This would be a non-invasive and easy diagnostic tool.

The study of tissue molecular profiles to better understand and diagnose disease pathophysiology has been examined in another common oral disease—periodontitis. Using biopsies from patients diagnosed with either chronic periodontitis or aggressive periodontitis, Kebschull et al. 49 explored whether molecular profiling can form the basis for a pathobiology-based classification that correlates with the phenotypic features of the disease. They identified two de novo clusters with high similarity in transcriptional profiles. The clusters had distinct molecular signatures, which did not align with the current classifications of periodontitis. However, they did translate into distinct phenotypic differences and can provide a basis for novel classification. This study along with current study supports the use of molecular profiling to classify disease.

Table 1. Selected upregulated or downregulated genes in pulpitis (Table 1) are expressed at relatively lower levels in pulp samples from asymptomatic to mild pain patient compared with those with moderate to severe pain. IL8, TNFA and IL1B have been shown to be potent mediators of pain. 34,35

IL8 has been recently associated with complex regional pain syndrome 36 and IL1B has been shown to contribute to the upregulation of nerve growth factor during inflammation that consequently induces inflammatory hyperalgesia. 37 Both IL8 and IL1B are expressed at relatively lower levels in zero to mild pain pulpitis patients. Several genes that are upstream on the inflammatory cascade of both IL8 and ILB (for example, TLR2 and TLR4) are significantly increased in pulpitis samples (Table 1). Furthermore, most genes associated with structural formation and integrity (for example, COL1-4 and MMPs) are expressed at relatively higher levels in pulpitis samples. In contrast, however, several genes that are known to be pain mediators or associated with increased pain experience did not have significant discordance between mild and moderate to severe pain (Table 1). For example, prosstaglandin-endoperoxide synthase 2 or cyclooxygenase-2, an important inflammatory enzyme that has been shown to be significantly elevated in pulpitis tissues, 38 did not have expression difference in mild pain but is significantly elevated in pulpitis compared with normal controls (Table 1).

IL8 showed the same results.

Pathogen-associated molecular pattern receptors, including TLRs, are key molecules in the response to pathological stimuli. Pulpitis is a disease that is mainly due to microbial insults. TLRs are expressed by both immune and non-immune cells in the pulp including neurons, fibroblasts, endothelial cells, epithelial cells and others. They recognize viral and microbial structures, as well as self molecules (such as single-stranded RNAs) that may accumulate in non-physiologic amounts or sites. 39,40 TLR ligands stimulate the production of pro-pain molecules and, in this way, may contribute to the pathogenesis of pain. 41,42 In this study, the expression of several TLRs (1, 2, 4, 6, 8 and 9) were upregulated and TLR3 was downregulated in inflamed pulps as compared with NPs. Our data also show that expression of TLR8 was higher in pulpitis patients experiencing severe pain as compared with those who reported no pain or mild pain. This is of particular interest given the recent finding that murine TLR7 (which is phylogenetically and structurally related to human TLR8) interacts with TRPA1 and that specific miRNAs activate nociceptors via TLR7 and TRPA1. 43–45

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Although microarray analysis is a powerful tool to study the simultaneous expression of several genes, it has some inherent limitations. These include technical factors such as limited dynamic range, signal saturations and cross-hybridization. It is important to also remember that the cellular profile of inflamed pulps differs from that of NPs. The residents cells in NPs include fibroblasts, odontoblasts and some immune cells such as macrophages, dendritic cells and mast cells. Inflamed pulps are characterized by an influx of immune cells and as such the results reported here may be in part, due to differences in the cellular makeup as well.

This study shows that there are differences in gene expression between normal and inflamed pulps and; between zero to mild and moderate to severe pulpitis pain. Among the genes reported to have a strong association with pain and inflammation, IL8 showed the highest fold change (35.9, q-value = 0) in pulpitis samples and it is significantly underexpressed in mild pain compared with moderate to severe pain (0.14, q-value = 0). IL8 is increased in gingival crevicular fluid from patients with acute pulpitis and may be a useful measurement for staging patients with acute pulpitis.9 The same trend was also noted in IL1A, IL1B and IL10RA.

This study provides a molecular basis for the clinical diagnosis of pulpsitis and for the differences or similarities between asymptomatic to mild pulpal pain vis-à-vis moderate to severe pain. With an enhanced understanding of pulp inflammation, future studies on treatment and management of pulpsitis and on pain associated with it can have a biological reference to bridge treatment strategies with pulpal biology.

**MATERIALS AND METHODS**

The study was approved by the University of North Carolina Office of Human Ethics. Eligible patients seeking treatment at the School of Dentistry were recruited to participate in the study. Informed consent was obtained from each participant. Demographic details, history of odontogenic pain, vital signs (blood pressure, pulse rate), medical history (including full details of any medication) and patient's smoking and oral habits were recorded.

Patient selection and pulpal diagnosis

The inclusion criteria were adults (18 years or older) presenting for endodontic treatment with no evidence of periapical pathoses (that is, radiolucency, swelling and pressure sensitivity) and no previous pulp therapy (that is, pulp capping, etc). Exclusion criteria were conditions requiring antibiotic prophylaxis or additional treatment procedures, debilitating disease, chronic pain, diabetes mellitus, hematological disorders or a history of taking centrally acting drugs (for example, tricyclic antidepressants) known to interfere with the release of various pain mediators and/or modify pain experience within the previous 6 months or over-the-counter pain medicine within the last 6 h, chronic use of medication known to affect the immune response and patients who were immunocompromised. Teeth with incompletely developed roots were also excluded.

Diagnosis of NP and IP was based on subjective and objective findings and was in accordance with the AAE guidelines. In brief, NP is a clinical diagnostic category in which the pulp is symptom-free and normally responsive to diagnostic testing. On the other hand, IP is clinical diagnosis indicating that the vital, inflamed pulp is incapable of healing. The symptoms include lingering and exacerbated pain in response to thermal stimuli, spontaneous pain, referred pain or no clinical symptoms but inflammation is evident after caries excavation or trauma. Pain history, cold test (Endo Ice Refrigerant Spray, Coltene Inc, Cuyahoga Falls, OH, USA) and electric pulp testing (Vitality Scanner 2006, Sybron Endodontics, Orange, CA, USA) were used to determine pulpal diagnosis. Percussion, palpation, probing, mobility and radiographs were used to rule out periapical pathosis.

Pain levels were assessed using a VAS. If lingering pain is present or significantly evoked by a stimulus compared with a control tooth, the patient was asked to quantify the intensity of pain by placing a mark on a 10-cm VAS with anchors of ‘no pain’ and ‘worst pain imaginable’. Using a modified version of the VAS recommended by Jensen et al., a VAS mark of ≤ 30 mm was classified under mild, 31–74 mm under moderate and 75–100 under severe pain.

A total of 40 pulp samples from 28 patients with IP and from seven patients with NP were included in the study. Twelve of the IP samples presented with moderate to severe pain at the time of pulp extirpation. The total number of samples was based on a recently published paper by our group on the differences of micro-RNA expression between normal and inflamed pulps and on a power analysis. With 20 samples in each group, we had 80% power to detect an effect size of 0.9 using a threshold of P < 0.05 for significance. If we required a more stringent threshold of P < 0.001, then we would have 80% power to detect an effect size of 1.4. Given that large effect sizes are common in microarray experiments,20 we had adequate power to detect the genes that exhibited the largest expression differences between the two groups. Moreover, we increased the power as much as needed by increasing the acceptable false discovery rate. Given that this initial analysis is intended to be exploratory, a higher false discovery rate is acceptable in this study. Thus, this study had adequate power to detect the genes with the largest group differences.

Sample collection

The inflamed pulps were extirpated during root canal treatment and the NPs were collected right after tooth extraction for orthodontic or restorative purposes or for third molar removal. Pulp collection methods were adapted from Awawdeh et al.31 with modifications. Briefly, local anesthesia (2% lidocaine with 1:100 000 epinephrine) was administered and an access cavity was prepared under rubber dam isolation. Pulp tissue was extirpated using a sterile barbed broach or Hedström-file, placed into a pre-weighted Eppendorf tube, immediately frozen in liquid nitrogen and stored at −70 °C. The root canal treatment was then completed. For the controls and for some patients who chose to have extraction instead of root canal treatment, all extractions were performed under local anesthesia. Immediately after extraction, each tooth were split in a vice fitted with a cutting edge and the pulp tissue was removed using a barbed broach or Hedström-file. The pulp was then placed in an Eppendorf tube, immediately frozen in liquid nitrogen and stored at −70 °C.

**RNA extraction and microarray analysis**

Total RNA was isolated using a commercial kit (Qiagen RNeasy Kit, Valencia, CA, USA) following the manufacturer's specifications. Quantity and purity of the total RNA was analyzed using Nano-Drop (Thermo Scientific, Wilmington, DE, USA) and Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Microarray experiments were conducted at the Microarray Core Facility of UNC-Chapel Hill Lineberger Cancer Center. The Nugen Ovation Pico WTA system V2 (San Carlos, CA, USA) and the Nugen Encore Biotin module were used to prepare RNA for hybridization onto Affymetrix GeneChip Human Gene 2.0 arrays (Santa Clara, CA, USA). Briefly, 25 ng of total RNA was converted into amplified SPiA complementary DNA using the Ovation Pico WTA system Biotin System Kit and the Nugen SPiA complementary DNA was purified using the Qiagen QIAQuick PCR purification kit. In all, 2.5 μg of SPiA complementary DNA was fragmented and labeled using the Nugen Encore Biotin module and accompanying user guide. The amplified SPiA complementary DNA was hybridized using the Qiagen QIAquick PCR purification kit. In all, 2.5 μg of SPiA complementary DNA was hybridized and labeled using the Nugen Encore Biotin module and accompanying user guide. The cocktail for hybridization onto an Affymetrix Human Gene 2.1 ST peg plate was prepared using the Nugen Ovation PicoSL WT System V2 user guide’s cocktail assembly table. The hybridization cocktails were denatured and hybridized on the Human Gene 2.1 ST peg plate in the Affymetrix GeneTitan MC Instrument. Washing and scanning was also carried out on the Affymetrix GeneTitan MC Instrument. Individual sample RNAs were hybridized individually to microarrays. Basic data analysis of the arrays was carried out using the Affymetrix Expression Console software. Gene expression levels between the control and diseased pulps were compared. Changes in gene expression levels were analyzed by GSEA (Broad Institute, Cambridge, MA, USA) and its leading edge analysis software that uses the permutation method.

The difference in gene expression levels between groups were determined by the significance analysis of microarray program using a false discovery rate (q-value) of 5%. As this study analyzed over 53 600 genes, only those that were previously reported to have key roles in pain and inflammation will be discussed in this article. In addition, only the key genes with a significance analysis of microarray fold change of over 1.5 (overexpressed) and under 0.5 (underexpressed) to signify a potentially relevant clinical effect are reported.36
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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