Profiling of MicroRNAs and Inflammatory Cytokines in Patients with External Cervical Root Resorption

2023 Biomedical Research Awards (BRA)

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FollowUp Form

Award Information

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)

- Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?

Title of Project:*
Profiling of MicroRNAs and Inflammatory Cytokines in Patients with External Cervical Root Resorption

Award Type
Biomedical Research Award (BRA)

Period of AAOF Support
July 1, 2023 through June 30, 2024

Institution
Indiana University

Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)
Rishma Shah (Principal Investigator), Glen Karunanayake (Co-investigator), Antonio Moretti (Co-investigator)

Amount of Funding
$40,000.00

Abstract
(add specific directions for each type here)

See attached file

Respond to the following questions:

Detailed results and inferences:*
If the work has been published, please attach a pdf of manuscript below by clicking "Upload a file".
<u>OR</u>
Use the text box below to describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis, and interpretation of results should also be attached by clicking "Upload a file".

Final Report for AAOF BRA ECR 30Jun2025 - Rishma Shah.pdf
Please see attached file for details of the study outcomes. Thank you.

Were the original, specific aims of the proposal realized?*
The original specific aims of the study are stated below and were realized upon completion of the study.

Aim 1: Investigation of miRNAs in ECR Lesions
We hypothesized specific miRNAs are associated with ECR lesions as compared to healthy control tissue. We identified specific miRNAs using reverse transcription quantitative real-time polymerase chain (RT-qPCR) reaction and explored potential miRNA targets of relevance to ECR.

Aim 2: Investigation of Inflammatory Markers in Gingival Crevicular Fluid Adjacent to ECR Lesions
We hypothesized specific inflammatory markers in gingival crevicular fluid (GCF) are associated with ECR compared to healthy control sites. We determined inflammatory marker levels within GCF using multiplex immmunoassays.

Were the results published?*
No

Have the results of this proposal been presented?*
Yes

To what extent have you used, or how do you intend to use, AAOF funding to further your career?*

AAOF funding has been invaluable in protecting my research time and supporting the generation of preliminary data to support applications for further funding. I have received one OFDFA and three BRAs from the AAOF - the outcomes from the studies supported by AAOF funds have helped me secure over $2m of funding from institutions, including an R01 award from the NIDCR.

Accounting: Were there any leftover funds?

$0.00

Not Published

Are there plans to publish? If not, why not?*

Yes, we are currently preparing a manuscript for the Journal of Endodontics.

Presented

Please list titles, author or co-authors of these presentation/s, year and locations:*

Seagroves JT, Ashraf N, Moretti A, Karunanayake GA, Shah R*. Profiling of Inflammatory Cytokines in External Cervical Resorption.
Presented at the American Association of Endodontists annual meeting 2024

Seagroves JT, Sun K, Portillo G, Liu C, Zou F, Ashraf N, Moretti A, Karunanayake GA, Shah R*. Micro-RNA and Inflammatory Cytokine Profiling in Patients in External Cervical Resorption.
Presented at the UNC-CH Adams School of Dentistry Research Day, 2025

Was AAOF support acknowledged?

If so, please describe:

AAOF support was acknowledged during both presentations, and will also be acknowledged in the manuscript to the Journal of Endodontics.

Internal Review

Reviewer comments

Reviewer Status*

File Attachment Summary

Applicant File Uploads

- Final Report for AAOF BRA ECR 30Jun2025 - Rishma Shah.pdf

**Profiling of MicroRNAs and Inflammatory Cytokines
in Patients with External Cervical Resorption
FINAL REPORT FOR AAOF BRA**

1. <u>Hypothesis and Specific Aims</u>

External cervical resorption (ECR) is a poorly investigated and potentially devastating dental disease due to frequent late presentation. Consequences include costly root canal treatment, or permanent tooth loss leading to complicated options for replacement. The ability to diagnose ECR at an early stage could allow for more simple treatments. In addition, a deeper knowledge of the etiology of ECR can enable the development of effective therapies.

In this interdisciplinary longitudinal cohort study, our aim was to investigate and confirm the presence of specific microRNAs (miRNAs) and inflammatory markers in teeth affected by ECR. Our study outcomes have the potential to add valuable knowledge to a poorly studied topic of interest to endodontists, orthodontists, periodontists, and prosthodontists who must manage the consequences of this devastating condition. Our **long-term goal** is to develop diagnostic/prognostic tests and novel miRNA-targeted therapies for ECR.

Our **hypothesis** was that differences exist in the expression of miRNAs and inflammatory cytokines in lesions associated with ECR as compared to control non-diseased teeth. The following **specific aims** were completed to answer our hypothesis:

- **Aim 1: Investigation of miRNAs in ECR Lesions**
  We hypothesized specific miRNAs are associated with ECR lesions as compared to healthy control tissue. We identified specific miRNAs using reverse transcription quantitative real-time polymerase chain (RT-qPCR) reaction and explored potential miRNA targets of relevance to ECR.

- **Aim 2: Investigation of Inflammatory Markers in Gingival Crevicular Fluid Adjacent to ECR Lesions**
  We hypothesized specific inflammatory markers in gingival crevicular fluid (GCF) are associated with ECR compared to healthy control sites. We determined inflammatory marker levels within GCF using multiplex immmunoassays.

2. <u>Subjects and Methods</u>

<u>Subjects</u>

Patients meeting the inclusion and exclusion criteria below were recruited from the Graduate and Dental Faculty Practice Endodontic and Periodontology clinics at the University of North Carolina at Chapel Hill Adams School of Dentistry (ASoD).

<u>Inclusion Criteria</u>

- Patients ≥ 18 years old
- Clinical and radiographic (CBCT) evidence of external root resorption in any tooth or jaw requiring treatment

- Good general health (ASA I/II)
- Able/willing to return 3 months after treatment for follow-up

Exclusion Criteria

- Any local or systemic conditions that affected the hard and soft tissues of the oral cavity
- Poor oral hygiene
- Regular use of medications known to affect the gingival and periodontal status (e.g. phenytoin, nifedipine, inhaled corticosteroids, etc.)
- Other types of root resorption present concurrently on same tooth (e.g. external apical root resorption)
- Tobacco users
- Pregnant women

After obtaining consent, the following data were collected:

- Medical and dental history
- Dental history of orthodontics, trauma, bleaching, periodontal graft, etc.
- Probing depths, clinical attachment levels, and bleeding on probing
- Mobility of involved tooth/teeth
- Clinical and radiographic imaging of the involved site, including CBCT
- Periodontal and endodontic diagnoses
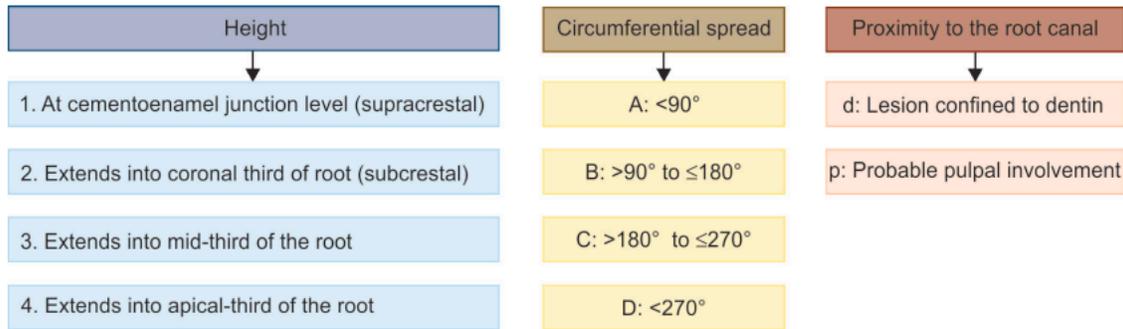- Treatment options
- ECR Classification (Table 1)

Height	Circumferential spread	Proximity to the root canal
1. At cementoenamel junction level (supracrestal)	A: <90°	d: Lesion confined to dentin
2. Extends into coronal third of root (subcrestal)	B: >90° to ≤180°	p: Probable pulpal involvement
3. Extends into mid-third of the root	C: >180° to ≤270°	
4. Extends into apical-third of the root	D: <270°	

Table 1: Classification for teeth affected by ECR
(Adapted from Patel *et al*., 2018)

Tissue Biopsy

Treatment of ECR involved access via a gingival flap and curettage of the tissue immediately adjacent to the resorption site. Patients were anesthetized and a tissue flap extending across multiple teeth was elevated gently to reveal the ECR lesion of the involved tooth. A periodontal curette was used to remove and place the tissue (~2-3mm^3) adjacent to the lesion into a tube containing 0.5 mL RNA*later*™ Stabilization Solution (Invitrogen™). Control tissue in the form of a connective tissue biopsy (~2-3mm^3) was obtained from the gingival flap furthest from the ECR-involved tooth. Biopsies were stored at -80ºC until the time of processing.

Biopsies were homogenized and the miRNeasy kit (QIAGEN) was used as per the manufacturer's instructions for miRNA and total RNA purification. RNA quantification and integrity were carried out using a NanoDrop (Thermo Scientific) and then cDNA synthesis by reverse transcription of 5ng/μl RNA was undertaken using the miRCURY LNA RT Kit (QIAGEN). The cDNA was used on two different miRCURY LNA miRNA PCR Panels (QIAGEN; Cat. No YAHS-211Y and YAHS-204Y) in combination with a miRCURY LNA SYBR Green Master Mix. CT values were exported to an Excel file to create a table of CT values. This table was then uploaded onto the data analysis web portal at http://www.qiagen.com/geneglobe. Samples were assigned to a Control Group or Test Group. CT values were normalized based on the geNorm (Pre-Defined Reference miRNAs Only) method. The data analysis web portal calculated fold change/regulation using the delta-delta CT method, in which delta CT is calculated between miRNA of interest and an average of reference miRNAs, followed by delta-delta CT calculations (delta CT (Test Group)-delta CT (Control Group)). Fold Change was then calculated using the $2^{\wedge}$ (-delta-delta CT) formula. Fold-change values greater than one indicated a positive- or an up-regulation, and fold-change values less than one indicated a negative or down-regulation. The paired Student's t-test was used with p values less than 0.05 indicating statistical significance.

Gingival Crevicular Fluid

GCF samples were obtained from the buccal and palatal surfaces of ECR-affected teeth and corresponding control healthy teeth in the contralateral quadrant of the same arch. The procedure of GCF sampling was based on the protocol recommended by Barros *et al.* (2016). Briefly, supragingival plaque and saliva around the teeth of interest were removed using an air-water syringe and cotton pellet. The teeth were then isolated with cotton rolls. Paper strips (PerioPaper, Oraflow Inc.) were used to collect GCF by gentle insertion into the gingival sulcus for up to 30 seconds. Strips visibly contaminated with blood or saliva were discarded. A calibrated Periotron® instrument (Oraflow Inc.) was used to record electrical capacitance of each paper strip. The actual volume was calculated at a later date from the standard curve obtained using known volumes of distilled water. For our subjects, GCF was collected on multiple paper strips until approximately ~4.8μl of fluid was obtained. The strips were wrapped in sterile foil, labelled with a code to ensure subject anonymization, and immersed in liquid nitrogen. Samples were transferred to a -80°C freezer for long-term storage until analysis. GCF collection occurred before probing and other clinical measurements in order to limit contamination of paper strips.

At the time of analysis, paper strips were transferred to sterile microcentrifuge tubes and GCF was eluted by addition of 180μL of assay diluent. The tubes were shaken at 180rpm for 20 minutes and then centrifuged at 700xg for 10 minutes. The supernatant was transferred to an array plate and stored at -80°C. Prior to carrying out the assay, samples were thawed completely and vortexed to mix thoroughly.

Cytokine levels were quantified using the highly sensitive Invitrogen Human Cytokine Magnetic 10-Plex Panel (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. Assay wells were pre-wetted with Working Wash Solution, and following incubation for 30 seconds at room temperature, the solution was decanted. A diluted solution of microsphere beads coated with specific antibodies against 10 target analytes was prepared, and 25μL of the solution was added to each well. The plate was protected from light for the

remainder of the process. 200µL of Working Wash Solution was then added to each well and the beads were allowed to soak for 30 seconds. Thereafter, the solution was removed and the plate blotted on clean paper towels to remove any residual liquid. The wash step was repeated.

Each sample was added to the panel in duplicate providing two technical repeats. A serial dilution of a known concentration of each protein of interest was also added to the panel to create a standard curve. 50µL of Incubation Buffer was pipetted into each well followed by 100µL of the appropriate standard dilution or 50µL of sample + 50µL Assay Diluent. The plates were covered and incubated for 2 hours at room temperature. After the incubation period, liquid was removed from all the wells, and the same wash step as above was used. 100µL of prepared 1X Biotinylated Detector Antibody was added, and wells were incubated for 1 hour at room temperature. Following another wash step, 100µL of prepared 1X streptavidin conjugated to the fluorescent protein R-phycoerythrin (Streptavidin-RPE) was added and incubated at room temperature for 30 minutes. Wells were then washed to remove unbound reagents and dried. 100µL of Working Wash Solution was added to resuspend the beads, and the Luminex xMAP System was used to analyze the samples. The standard curve was used to determine the absolute concentration of cytokine in a sample, which in turn was normalized to the total volume of GCF collected for that particular sample.

The Wilcoxon signed-rank test was used to compare cytokine levels between healthy teeth and teeth with ECR at a significance level of $p < 0.05$. Analyses were performed using R (Version 4.4.0, http://www.r-project.org).

A ratio of expression between different cytokines in diseased and healthy teeth of the same individual was also calculated. This approach was based on a similar study in an attempt to reduce any bias and variability due to generalized gingivitis or periodontitis (Nunez *et al*., 2023). This adjusted cytokine value (ACV) was calculated by dividing the cytokine concentration in the ECR-affected tooth by the cytokine concentration in the control tooth. This was calculated for all cytokines from each tooth sampled. An ACV>1 indicated detection of a greater cytokine level in the diseased tooth. The percentage of teeth with ACV>1 was compared for each cytokine.

3. Results and Discussion

Demographic, Clinical Data, and ECR Management Plan

Subject ages ranged from 41-83 years with a gender distribution of 7 women and 5 men. ECR lesions were evenly distributed between canine, premolars, and molars with 3 teeth in each group. All study teeth had pulpal and periapical diagnoses of normal pulp and normal periapical tissues respectively. There were varied periodontal diagnoses ranging from gingival health to periodontitis. Subject demographic data is summarized in Table 2. The most common possible predisposing factor for ECR identified was orthodontic treatment. Other factors included trauma, cracks, parafunctional habits, and extraction of an adjacent tooth.

With respect to the CBCT findings, it was noted the lesion height was within the coronal or middle third of the root in 92% of affected teeth. The circumferential spread was ≤90° in 43% of teeth, and >90° to ≤180° in 50% of teeth. 92% of teeth had probable pulpal involvement.

	Female	Male	Total
n	7	5	12
Age (mean (se))	63.67 (10.41)	62 (13.15)	62.75 (10.64)
Incisor affected	0	3	3
Canine affected	3	1	3
Premolar affected	3	0	3
Molar affected	1	2	3
Pulpal diagnosis: normal pulp	7	5	12
Periapical diagnosis: normal periapical tissues	7	5	12
Periodontal diagnosis: gingivitis	4	2	6
Periodontal diagnosis: gingival health on a reduced periodontium	1	1	2
Periodontal diagnosis: gingivitis on a reduced periodontium	1	0	1
Periodontal diagnosis: periodontitis*	1	2	3
Height: at CEJ or supracrestal (1)	1	0	1
Height: extends into coronal third of root or subcrestal (2)	3	1	6
Height: extends into middle third of root (3)	3	2	5
Height: extends into apical third of root (4)	0	0	0
Circumferential spread: ≤90° (A)	2	3	5
Circumferential spread: >90° to ≤180°	4	2	6
Circumferential spread: >180° to ≤270°	1	0	1
Circumferential spread: >270°	0	0	0
Proximity to root canal: confined to dentine (d)	0	1	1
Proximity to Root Canal: probable pulpal involvement (p)	7	4	11

Table 2: Age, Teeth Affected, Endodontic Diagnoses, Periodontal Diagnoses, and Patel 3D Classification of ECR Lesion Extent Measured by CBCT of Subjects with ECR
* Periodontitis patients had the following diagnoses: Stage 1 Grade B Generalized, Stage 3 Grade B Generalized, Stage 3 Grade C Localized

Expression of miRNAs

A total of four tissue samples were obtained from ECR-affected teeth that were restored. Control tissue biopsies were also obtained. Table 3 outlines the miRNAs that were found to be significantly *down-regulated* in the tissue within ECR lesions, and their possible role in ECR. There were no miRNAs that were found to be significantly up-regulated in the ECR tissue.

miRNA	Fold Regulation	p-Value	Possible Role in ECR
hsa-miR-205-5p	-3.66	0.005918	Down-regulates the following genes involved in bone formation: • RUNX2, a transcription factor for the differentiation of osteoblasts. • NOTCH2, a regulator involved in bone angiogenesis.
hsa-miR-23b-3p	-2.13	0.015147	Down-regulates RUNX2, a transcription factor for the differentiation of osteoblasts.
hsa-miR-27a-3p	-1.61	0.017164	Down-regulates the following genes that negatively regulate dental tissue regeneration: • DKK3, a negative regulator of the Wnt signaling pathway. This leads to activation of the Wnt pathway leading to odonto/osteogenic differentiation of dental pulp stem cells. Activation of the Wnt pathway leads to increased expression of RUNX2. • SOSTDC1, a negative regulator of the BMP signaling pathway. This leads to activation of the pathway, which together with Wnt pathway activation, leads to greater differentiation of dental pulp stem cells into cells that can form hard tissue. • AXIN2, a negative regulator of the Wnt/β-catenin signaling pathway. With activation of the pathway, there is odonto/osteogenic differentiation of dental pulp cells. • APC, a suppressor of the canonical Wnt signaling pathway, which then leads to Wnt activation and enhanced odonto/osteogenic differentiation.
hsa-miR-27b-3p	-1.74	0.011737	• Involved in inhibition of ameloblast differentiation. • Down-regulates BMPR1A leading to suppression of osteogenic and odontogenic differentiation of dental pulp stem cells.

Table 3: miRNAs found to be significantly differentially expressed within the tissue of ECR lesions compared to control tissue.

The miRNAs found to be significantly down-regulated in the ECR tissue are involved in dental and bone regeneration. Down-regulation of hsa-miRNA-205-5p and hsa-miRNA-23b-3p leads to expression of genes associated with bone regeneration suggesting that the tissue within the ECR lesion may be under repair. Although down-regulation of hsa-miR-27b-3p may lead to promotion of dental tissue regeneration, down-regulation of hsa-miR-27a-3p results in up-regulation of multiple genes that negatively regulate dental tissue regeneration. Therefore, it could be inferred any attempt at repair of the ECR lesion is undertaken by cells other than those from the dental pulp. With the miRNAs identified playing a significant role in dental tissue regeneration and hard tissue formation, we plan to investigate them further in potential therapeutic applications.

Cytokine Expression in the GCF

A total of 24 GCF samples were collected from ECR-affected teeth (N=12) and control teeth (N=12). Figure 1 shows the distribution of cytokine levels measured in GCF of teeth with ECR and healthy control teeth, showing significant variability between subjects and between cytokines. The cytokines with the greatest relative abundance across all subjects were IL-8 followed by IL-1β, while GM-CSF, IL-5, IFN-γ, and IL-2 were detected in low levels. IL-6, TNF-α, IL-10, and IL-4 showed moderate relative expression. Figure 2 shows the per cent of subjects with an ACV > 1, indicating a greater cytokine concentration in the subject's diseased tooth compared to their healthy tooth. 67% of subjects had an ACV > 1 for pro-inflammatory cytokines IL-1β and IL-8, and anti-inflammatory cytokines IL-10 and IL-4.

Figure 1: Distribution of cytokine concentrations (pg/mL) in GCF from diseased and control teeth. Each dot represents an individual cytokine measurement in the GCF of a single tooth (D represents teeth with ECR/disease, while C represents healthy teeth/control). Black bars represent the median concentration of each cytokine.
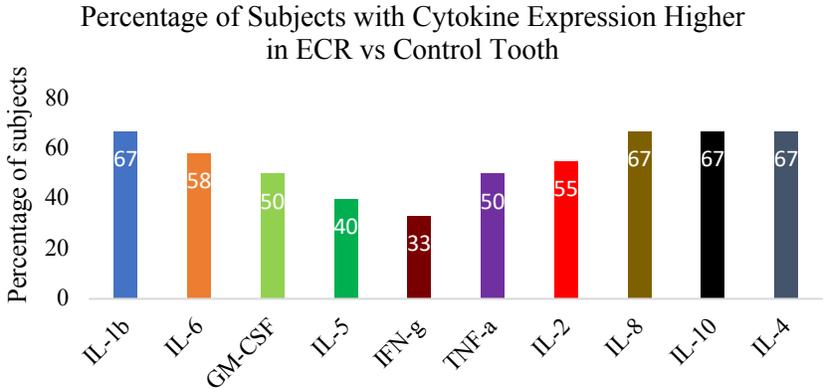

Figure 2: Percentage of subjects with a ratio of ECR tooth cytokine level to control tooth cytokine level greater than 1 (adjusted cytokine value; ACV > 1) indicating greater expression of cytokines in diseased versus healthy teeth.

Table 4 shows the mean GCF cytokine levels (pg/ml) for the entire group while Table 5 shows the levels stratified by gender (F=7, M=5). The cytokines with the greatest expression across all teeth were IL-1β and IL-8. IL-1β levels were higher in the GCF of diseased teeth versus control teeth (52 pg/ml vs 35 pg/ml) with borderline statistical significance (p=0.06). IL-8 and IL-10 were also expressed at greater mean levels in teeth with ECR while levels of IL-6 were reduced compared to healthy controls, although these results were not statistically significant. All cytokines except IL-4 showed greater mean expression in female compared to male subjects.

		Control (n=12) Mean ± SE	ECR (n=12) Mean ± SE	P value
Pro-inflammatory	IL-1β	34.51 ± 9.41	51.59 ± 13.48	0.06
	IL-6	11.61 ± 3.79	8.00 ± 1.64	0.96
	GM-CSF	0.65 ± 0.31	0.87 ± 0.40	0.83
	IL-5	0.28 ± 0.12	0.42 ± 0.26	1
	IFN-γ	0.80 ± 0.32	1.20 ± 0.51	0.81
	TNF-α	3.04 ± 0.62	3.74 ± 0.88	0.85
	IL-2	0.96 ± 0.15	1.27 ± 0.33	0.82
	IL-8	2177.14 ± 674.64	3466.78 ± 1261.32	0.62
Anti-inflammatory	IL-10	2.26 ± 0.75	3.42 ± 1.18	0.13
	IL-4	5.16 ± 1.55	6.69 ± 1.90	0.19

Table 4: Mean (standard error) Cytokine Expression (pg/mL) in Control and ECR Teeth

		Female Control (n=7) Mean ± SE	Female ECR (n=7) Mean ± SE	Male Control (n=5) Mean ± SE	Male ECR (n=5) Mean ± SE
Pro-inflammatory	IL-1β	37.34 ± 16.55	63.71 ± 20.80	31.11 ± 8.40	34.63 ± 12.93
	IL-6	15.95 ± 6.44	10.17 ± 2.45	6.41 ± 2.06	4.96 ± 1.14
	GM-CSF	0.93 ± 0.54	1.37 ± 0.64	0.31 ± 0.21	0.19 ± 0.12
	IL-5	0.38 ± 0.20	0.71 ± 0.42	0.15 ± 0.10	0.01 ± 0.01
	IFN-γ	1.15 ± 0.54	1.86 ± 0.80	0.39 ± 0.17	0.27 ± 0.13
	TNF-α	3.32 ± 1.09	4.74 ± 1.39	2.71 ± 0.53	2.35 ± 0.45
	IL-2	0.94 ± 0.27	1.60 ± 0.55	0.99 ± 0.14	0.81 ± 0.10
	IL-8	2694.97 ± 1179.13	4997.35 ± 2007.37	1555.75 ± 490.68	1323.99 ± 264.99
Anti-inflammatory	IL-10	2.59 ± 1.37	4.86 ± 1.88	1.86 ± 0.42	1.42 ± 0.20
	IL-4	4.11 ± 1.62	6.40 ± 2.27	6.43 ± 2.93	7.09 ± 3.59

Table 5: Mean (standard error) Cytokine Expression (pg/mL) in Control and ECR Teeth by Sex

Table 6 displays the mean cytokine level (pg/ml) in relation to the extent of the ECR lesion. As the lesion extended apically, there was a corresponding increase in mean levels of IL-1β, IFN-γ, TNF-α, IL-2, IL-8, and IL-10. As the lesion extended in a circumferential direction, there was also a corresponding increase in mean levels of IL-1β, TNF-α, IL-2, IL-8, IL-10, and IL-4. These results were not significant but show a general trend of increased cytokine expression with increased radiographic involvement of ECR lesions.

Table 7 shows mean cytokine level (pg/ml) for the type of tooth (incisor, canine, premolar, molar) affected by ECR. Mean levels of IL-1β and IL-8 were highest in molar teeth and lowest in incisors.

Figures 3-12 depict mean levels of the individual cytokine of interest in the total subject groups as a function of gender and lesion extent in individual planes of space as per the Patel 3D classification of ECR.

	Height				Circumferential Spread				Proximity to Root Canal	
	1 (n=1)	2 (n=6)	3 (n=5)	4 (n=0)	A (n=5)	B (n=6)	C (n=1)	D	d (n=1)	p (n=11)
Pro-inflammatory										
IL-1β	72.34	23.80 ± 10.61	80.79 ± 23.74	-	28.69 ± 14.93	60.60 ± 20.61	112.07	-	72.53	49.69 ± 14.61
IL-6	5.35	9.62 ± 2.99	6.59 ± 1.66	-	7.61 ± 3.92	8.06 ± 1.24	9.60	-	4.82	8.29 ± 1.77
GM-CSF	0.48	0.06 ± 0.06	1.93 ± 0.76	-	0.19 ± 0.12	1.30 ± 0.75	1.77	-	0.37	0.92 ± 0.44
IL-5	0.07	0.00 ± 0	0.99 ± 0.55	-	0.01 ± 0.01	0.79 ± 0.49	0.23	-	0.00	0.46 ± 0.28
IFN-γ	0.36	0.25 ± 0.12	2.51 ± 0.99	-	0.32 ± 0.12	1.75 ± 0.96	2.30	-	0.62	1.25 ± 0.56
TNF-α	2.68	1.93 ± 0.28	6.13 ± 1.58	-	2.46 ± 0.44	4.44 ± 1.65	5.99	-	3.04	3.81 ± 0.96
IL-2	0.43	0.79 ± 0.23	2.01 ± 0.64	-	0.58 ± 0.17	1.80 ± 0.57	1.54	-	0.62	1.33 ± 0.36
IL-8	1739.23	962.25 ± 227.57	6817.73 ± 2363.08	-	1184.89 ± 326.76	4811.52 ± 2308.09	6807.81	-	1874.96	3611.49 ± 1372.58
Anti-inflammatory										
IL-10	1.19	1.49 ± 0.24	6.19 ± 2.42	-	1.28 ± 0.25	3.60 ± 1.50	13.05	-	1.48	3.60 ± 1.28
IL-4	1.97	6.70 ± 3.09	7.61 ± 2.85	-	2.42 ± 1.34	10.08 ± 3.05	7.68	-	2.22	7.09 ± 2.03

Table 6: Mean (standard error) Cytokine Expression (pg/ml) of Teeth with ECR by Radiographic Extent in Different Planes of Space)
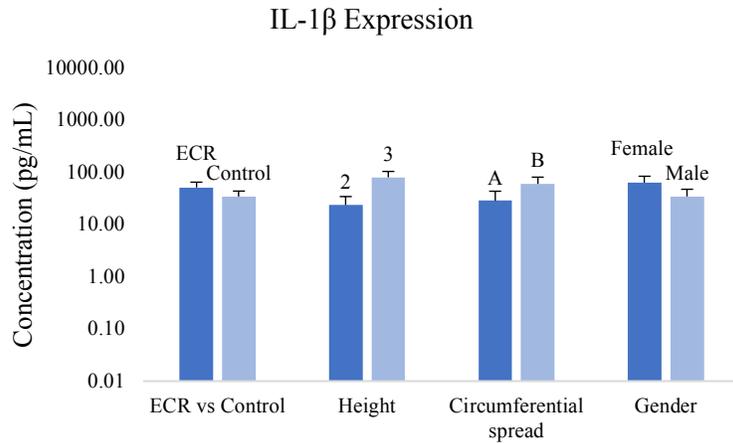
IL-1β Expression

Figure 3: IL-1β Expression by ECR Presence, Radiograph Extent, and Gender. Borderline significant difference (p=0.064) in expression between ECR and Control. Trend of increased expression with increasing radiographic extent and female gender.
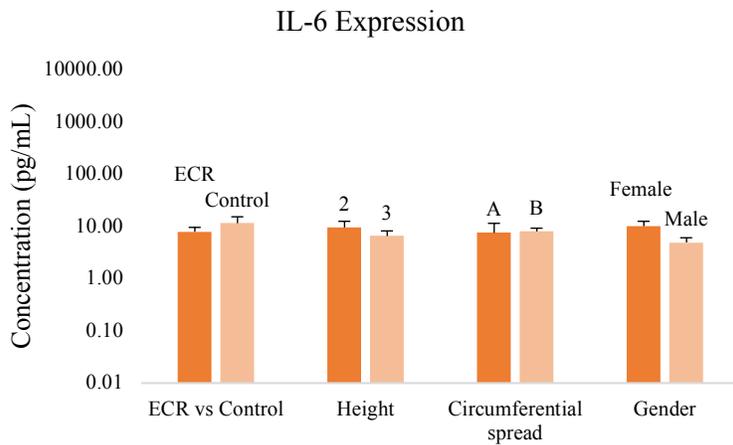
IL-6 Expression

Figure 4: IL-6 Expression by ECR Presence, Radiograph Extent, and Gender. Trend of increased expression in control teeth and female gender.
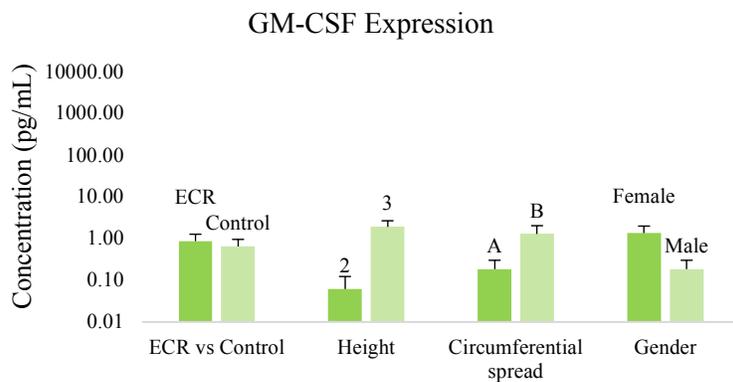
GM-CSF Expression

Figure 5: GM-CSF Expression by ECR Present/Absent, Radiograph Extent, and Gender. Trend of increased expression with increasing radiographic extent and female gender.
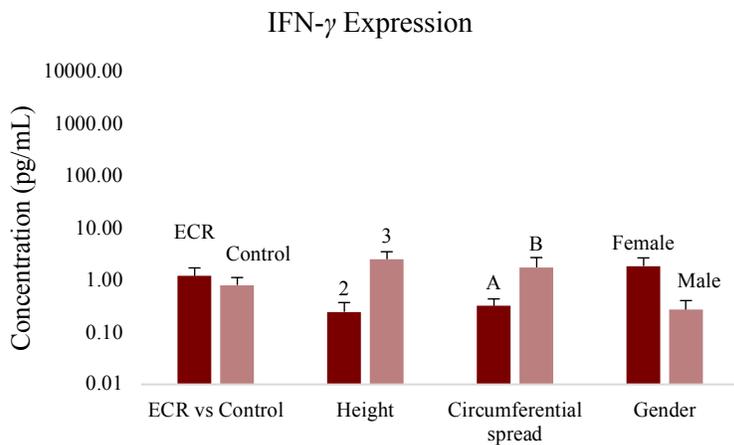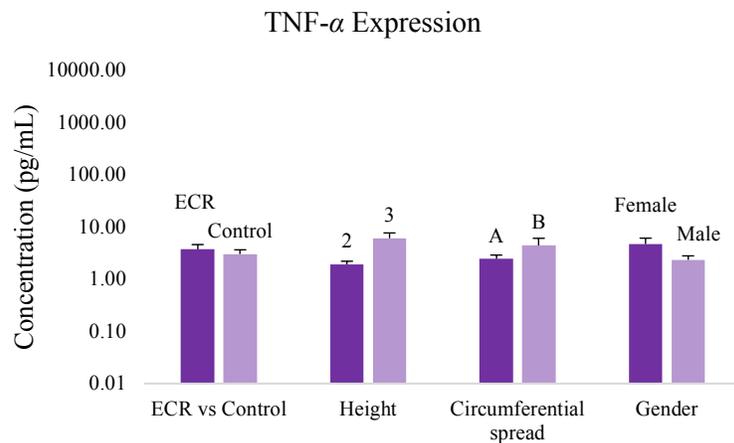
IL-5 Expression

Figure 6: IL-5 Expression by ECR Present/Absent, Radiograph Extent, and Gender. Trend of increased expression with increasing radiographic extent and female gender.

IFN-γ Expression

Figure 7: IFN-γ Expression by ECR Present/Absent, Radiograph Extent, and Gender. Trend of increased expression with increasing radiographic extent and female gender.
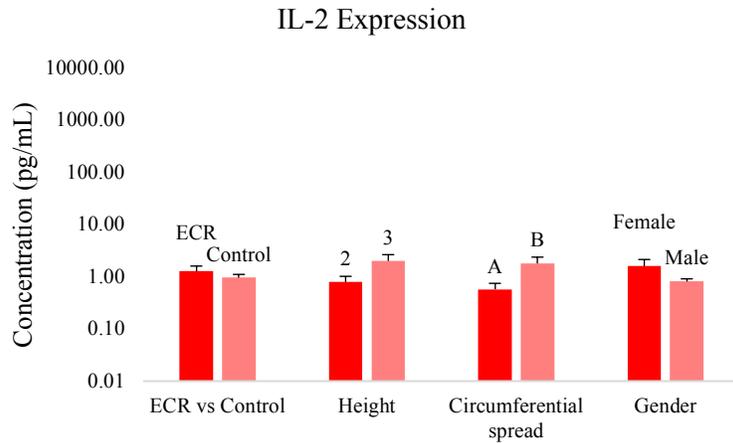
TNF-α Expression

Figure 8: TNF-α Expression by ECR Present/Absent, Radiograph Extent, and Gender. Trend of increased expression with increasing radiographic extent and female gender.

Figure 9: IL-2 Expression by ECR Present/Absent, Radiograph Extent, and Gender. Trend of increased expression with increasing radiographic extent and female gender.
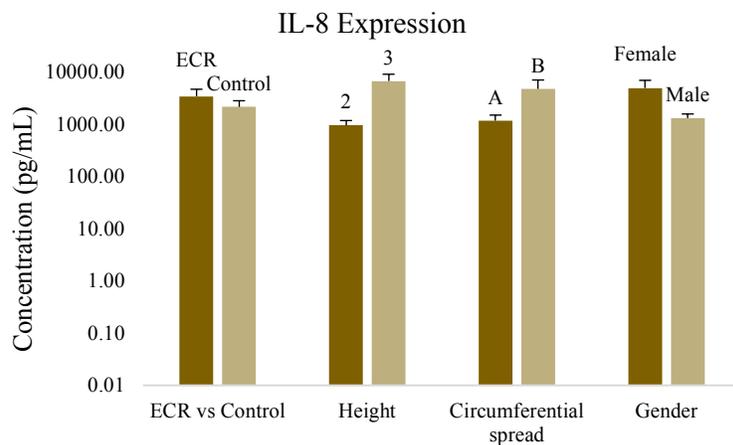

Figure 10: IL-8 Expression by ECR Present/Absent, Radiograph Extent, and Gender. Increased expression in teeth with ECR versus control (nonsignificant, p=0.62). Trend of increased expression with increasing radiographic extent and female gender.
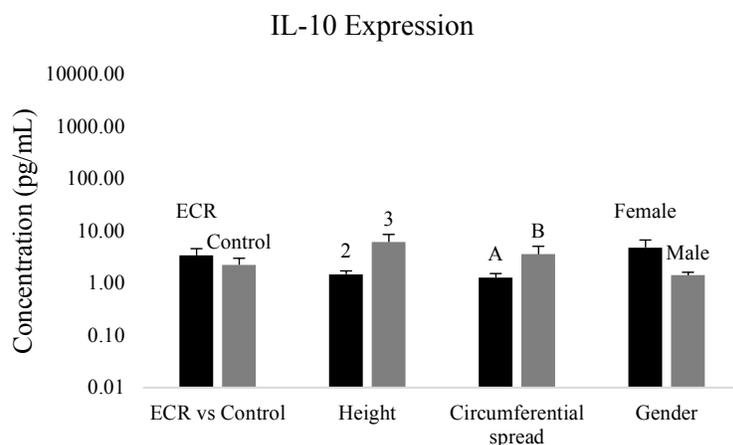

Figure 11: IL-10 Expression by ECR Present/Absent, Radiograph Extent, and Gender. Increased expression in teeth with ECR versus control (nonsignificant, p=0.12). Trend of increased expression with increasing radiographic extent and female gender.
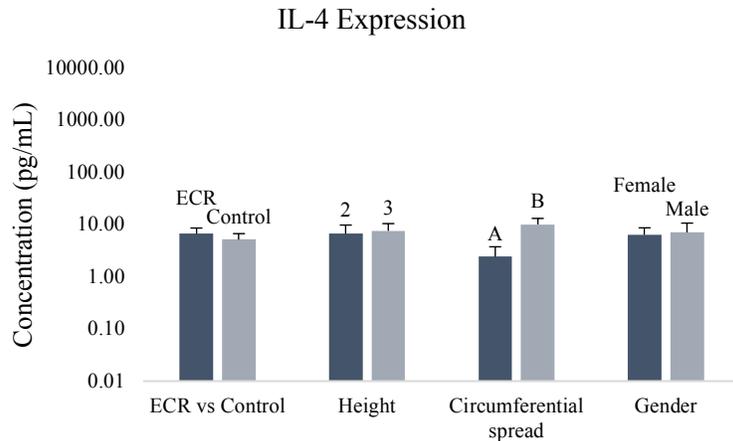
IL-4 Expression

Figure 12: IL-4 Expression by ECR Present/Absent, Radiograph Extent, and Gender. Increased expression in teeth with ECR versus control (nonsignificant, p=0.19). Trend of increased expression with increasing radiographic extent.

In general, the innate immune response of the periodontium represents the first line of defense. It involves nonspecific identification of a stimulus antigen via chemotactic recruitment of macrophages, neutrophils, and natural killer cells. This is followed by the adaptive immune response with influx of T- and B cells. Pro-inflammatory cytokines can have stimulatory influences on both responses, and affect the periodontium by promoting cellular recruitment and hard tissue resorption. With removal of the stimulus, there is a switch in phenotype of the immune cells to an anti-inflammatory state involving the recruitment of cells involved in tissue repair and elaborating cytokines involved in healing.

In our study, we found a general trend of increased expression of IL-1β, IL-8, and IL-10 in teeth affected by ECR as compared to control healthy teeth. IL-1β is a potent upregulator of osteoclastogenesis and bone resorption via the RANK/RANKL/OPG pathway, and is a frequently studied cytokine in periodontology and its relationship to periodontal and peri-implant bone resorption (Almehmadi and Alghamdi, 2018). Increased expression of IL-1β has been found in saliva of patients with orthodontically-induced external surface resorption, in GCF of teeth with external inflammatory resorption, and in homogenized root fragments of teeth with external replacement resorption (Bastos *et al*., 2017; Yashin *et al*., 2017; Gregorczyk-Maga *et al*., 2019). IL-8 also promotes RANKL expression by osteoblasts, thus upregulating osteoclastogenesis and bone resorption (Bendre *et al*., 2003). Other functions include neutrophil chemotaxis and angiogenesis. IL-8 has been found at greater levels in teeth with external inflammatory resorption and external replacement resorption (Bastos *et al*., 2017), and in GCF of teeth undergoing continuous orthodontic forces (Kaya *et al*., 2010). The anti-inflammatory IL-10, on the other hand, inhibits osteoclastogenesis thereby decreasing the extent of bone loss secondary to apical periodontitis, and dampens the immune response and cytokine activity (Gazivoda *et al*., 2009). It has been found in greater levels in saliva of patients with external surface resorption of teeth undergoing orthodontic treatment (Yashin *et al*., 2017). Our investigation also found a nonsignificant increase in IL-4 levels in teeth affected by ECR. IL-4 is an anti-inflammatory cytokine produced by mast cells, eosinophils, basophils, and T cells. It has been shown to increase IL-10 production, and may be responsible for reducing the extent of root resorption (He *et al*., 2015).

During the initiation stage of ECR, macrophages are reportedly amongst the first cells to migrate to the area of PDL damage. The ensuing granulation tissue that forms ultimately contacts

exposed dentin to facilitate bone or immune cells to begin the resorptive process (Mavridou *et al*., 2016). Macrophages are known to release pro-inflammatory cytokines, such as IL-1β and IL-8, and anti-inflammatory cytokines, such as IL-10, during chronic phases of the innate immune response. Our finding of increased expression of IL-1β, IL-8, and IL-10 in GCF of teeth with ECR supports the role of macrophages in ECR pathogenesis. Previous studies have suggested that macrophage polarization may be important to the development of orthodontically-induced external surface resorption. M1-activated macrophages produce pro-inflammatory cytokines whereas M2-activated macrophages produce anti-inflammatory cytokines. Resorption was augmented by an increased M1/M2 ratio signifying a pro-inflammatory microenvironment, while it was attenuated by a reduced M1/M2 ratio (He *et al*., 2015). A balance of both M1- and M2-activated macrophages and their respective cytokines may therefore be significant to ECR.

IL-1β, IL-8, and IL-10 expression additionally increased with an increase in coronal-apical height and circumferential spread of ECR measured radiographically. It is possible that as the size of the ECR lesion increases, there is greater involvement of macrophages or other regulatory cells and a concomitant increase in pro-resorption cytokine production. Future studies should include more teeth with varying degrees of ECR involvement (this study included no teeth with ECR extending past the middle third of the root or ≥270° circumferential spread) to better ascertain the effect of lesion size on cytokine expression.

GCF volume and GCF-derived cytokines have been found to display a sex difference. Studies have found that males display greater cytokine expression overall due to greater concentration of immune cells (Beenakker *et al*., 2020), and thus expression of cytokines in GCF is also increased in males (Tavakoli *et al*., 2022). These findings are contrary to our study findings. In this study, female subjects expressed greater levels of all cytokines in teeth with ECR except IL-4. Whether or not the sex differences in cytokine expression found in this study is of clinical relevance is up for debate, as there did not appear to be any sex differences found in radiographic or clinical severity of ECR lesions.

One patient had their tooth with ECR extracted due to planned implant replacement as part of a full mouth rehabilitation, two patients received root canal treatment with surgical repair of the ECR lesion, and nine patients elected to monitor the tooth until it became symptomatic. There was no apparent relationship between ECR extent, clinical parameters such as endodontic or periodontal diagnoses, and the decision to treat versus monitor. The teeth that were treated had identical diagnoses and radiographic classification as some teeth that were monitored, therefore the decision to treat seemed to be made based on patient wishes and future restorative plans. All teeth that were elected to be treated were anterior teeth, while a majority (67%) of teeth to be monitored were posterior teeth. Additionally, teeth with ECR extending into the middle third of the root were always chosen to be monitored by patients and providers in this study cohort. All patients electing for treatment involving surgical repair presented to the Endodontics Clinic initially, while patients electing for monitoring of the lesion were generally from the Periodontology or General Dentistry Clinics. The patient electing for extraction was seen in the Periodontology Clinic where they were undergoing multiple extraction of teeth and implant placement.

Strengths of this study include its engagement of multiple modalities such as CBCT and GCF collection to analyze ECR in a diverse group of patients. Our general trends of higher IL-1β and IL-8 expression in GCF of diseased teeth are similar to findings of other investigators studying

their expression in GCF of teeth with periodontitis (Offenbacher *et al.*, 2007), external inflammatory resorption (Gregorczyk-Maga *et al.*, 2019), and apical periodontitis (Nunez *et al.*, 2023). In the future, we aim to compare cytokine expression of GCF in teeth with ECR before and after treatment to ascertain the effect of treatment on the pro-inflammatory state.

3. <u>Impact</u>

a. Grants

<u>In Preparation</u>
Feb 2026   NIDCR R21 Award
                "Development of a Drug Delivery System for the Treatment of External Cervical Resorption"
                PI: **Shah R**
                10% effort. Direct Costs: ~$275,000.

b. Publications and Presentations

- Seagroves JT, Moretti A, Karunanayake GA, **Shah R***. Profiling Cytokines in Gingival Crevicular Fluid of Teeth Affected by External Cervical Resorption.
  In preparation for the Journal of Endodontics
- Seagroves JT, Ashraf N, Moretti A, Karunanayake GA, **Shah R***. Profiling of Inflammatory Cytokines in External Cervical Resorption.
  Presented at the American Association of Endodontists annual meeting 2024
- Seagroves JT, Sun K, Portillo G, Liu C, Zou F, Ashraf N, Moretti A, Karunanayake GA, **Shah R***. Micro-RNA and Inflammatory Cytokine Profiling in Patients in External Cervical Resorption.
  Presented at the UNC-CH Adams School of Dentistry Research Day, 2025

4. <u>A Note of Gratitude</u>

Funding from the AAOF has been invaluable to support the purchase of research supplies and protection of my time to undertake the above outlined work. I am very grateful for the award and the multiple achievements that it has facilitated. Thank you.

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