AJMB
Avicenna Journal of Medical Biochemistry
Avicenna J Med Biochem 2025;13(2): 89-96. doi:10.34172/ajmb.2676
http://ajmb.umsha.ac.ir

Original Article

# Western Blotting of Salivary Interleukin-1β and Tumor Necrosis Factor-α for Non-Invasive Diagnosis of Orthodontically Induced Root Resorption

**Homa Farhadifard[1], Parnia Yari[1], Amir Taherkhani[2,3*], Vahid Siavashi[4], Parzhin Moradi[1]**

[1]Department of Orthodontics, School of Dentistry, Hamadan Dental Research Center, Hamadan University of Medical Sciences, Hamadan, Iran
[2]Research Center for Molecular Medicine, Institute of Cancer, Avicenna Health Research Institute, Hamadan University of Medical Sciences, Hamadan, Iran
[3]Urology and Nephrology Research Center, Avicenna Institute of Clinical Sciences, Hamadan University of Medical Sciences, Hamadan, Iran
[4]Azma Teb Gostar Sorena Research Company, Basic Medical Science Research Center, Tehran, Iran

**Article history:**
**Received:** June 14, 2025
**Revised:** October 28, 2025
**Accepted:** November 7, 2025
**ePublished:** December 31, 2025

***Corresponding author:**
Amir Taherkhani,
Emails: amir.007.taherkhani@gmail.com;
a.taherkhani@umsha.ac.ir

**Abstract**

**Introduction:** Orthodontically induced inflammatory root resorption (OIIRR) is an unpredictable side effect of orthodontic treatment, typically identified only after irreversible damage. This study sought to assess whether the salivary concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) could function as dependable diagnostic indicators for OIIRR. It was hypothesized that OIIRR patients would demonstrate higher salivary TNF-α and IL-1β than controls, and that these cytokines would be accurate diagnostic biomarkers.

**Methods:** A case-control study was conducted involving 24 orthodontic patients, 12 with radiographically verified OIIRR and 12 controls free of root resorption, comparable in age and gender. Whole saliva samples were obtained, and protein expression of IL-1β and TNF-α was quantified via Western blotting using β-actin as a reference. Statistical methods included independent-samples t-tests, Cohen's *d* for effect size estimation, and receiver operating characteristic (ROC) curve analysis.

**Results:** Salivary IL-1β was significantly higher in cases (1.36 ± 0.33) than in controls (0.68 ± 0.16; $t(22) = 6.32$, $P < 0.0001$; Cohen's $d = 2.58$). Likewise, TNF-α was notably elevated in cases (1.38 ± 0.29) versus controls (0.80 ± 0.15; $t(22) = 6.27$, $P < 0.0001$; Cohen's $d = 2.56$). Moreover, ROC analysis revealed outstanding diagnostic performance for both markers, with AUC values of 0.986 for IL-1β and 0.951 for TNF-α. Optimal cutoffs were also 0.875 and 1.1 for IL-1β and TNF-α, respectively.

**Conclusion:** Our findings indicated that both cytokines represent valuable non-invasive salivary biomarkers for the early diagnosis and monitoring of OIIRR, potentially supporting safer orthodontic care. Nonetheless, larger prospective studies are needed for confirmation.

**Keywords:** Orthodontic root resorption, Diagnostic, Biomarkers, Western blotting, Non-invasive

**Please cite this article as follows:** Farhadifard H, Yari P, Taherkhani A, Siavashi V, Moradi P. Western blotting of salivary interleukin-1β and tumor necrosis factor-α for non-invasive diagnosis of orthodontically induced root resorption. Avicenna J Med Biochem 2025;13(2): 89-96. doi:10.34172/ajmb.2676

## Introduction

Orthodontic treatment uses mechanical forces to remodel periodontal tissues, thereby shifting misaligned teeth into correct three-dimensional positions. Orthodontic tooth movement (OTM) is a sterile inflammatory process involving osteoclastic bone resorption on the compressed side and osteoblastic bone formation on the tension side (1). Nevertheless, this inflammatory process may also lead to orthodontically induced inflammatory root resorption (OIIRR), which is a frequent adverse effect of orthodontic treatment (2). OIIRR represents a highly intricate, non-infectious inflammatory reaction that encompasses numerous distinct elements, including applied mechanical forces, tooth structures, bone tissue, various cell types, the adjacent extracellular matrix, and a range of biochemical signaling molecules (3, 4). If unwanted root resorption could be avoided, orthodontic therapy would become simpler and safer (5).

Cytokines monitor and regulate inflammatory and immune responses through complex mechanisms and

© 2025 The Author(s); Published by Hamadan University of Medical Sciences. This is an open-access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

serve as disease biomarkers (6). Furthermore, they regulate growth, cell maturation, cytotoxic and allergic immunity, responsiveness, and eosinophilia (7). As central mediators during orthodontic tooth movement, cytokines are also secreted in response to mechanical stress when the periodontal ligament is comspressed on one side and stretched on the other (3). Additionally, many cytokines recruit and activate immune cells at the site of force application, initiating and controlling bone remodeling (8, 9).

Moreover, earlier investigations have highlighted the role of cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), in dental root resorption. In this context, Curl and Sampson (10) explored the reasons behind the inability of osteoprotegerin (OPG) to prevent orthodontic root resorption. and demonstrated that TNF-α, along with its receptor TNFR1, was localized in areas of sterile periodontal inflammation, particularly in OPG-treated rats. This observation indicates that TNF-α offers an alternative route for osteoclast formation, thereby bypassing OPG's inhibitory effects and perpetuating root resorption.

Saliva is a valuable, non-invasive biofluid for biomarker discovery, as it is easy to collect and causes minimal patient discomfort. In addition, it contains a diverse range of molecules, including DNA, RNA, proteins, cytokines, hormones, metabolites, exosomes, and microbial signatures, that reflect both local oral health and systemic physiology. As such, saliva serves as a "mirror" of health and disease, offering great potential for early detection, risk assessment, and point-of-care decision making across oral and systemic conditions (11-14).

Additionally, Western blot is a foundational protein analysis technique that combines gel electrophoresis (size separation) with antibody-based detection, providing high specificity and sensitivity for identifying target proteins, even in complex mixtures at low abundance (15). The key advantages of this technique include a two-step recognition process that minimizes false positives compared to enzyme-linked immunosorbent assay (ELISA), making it particularly valuable in applications such as human papillomavirus serologic diagnosis (16). Due to its specificity, this technique also remains a valuable orthogonal method for validating salivary protein markers.. Furthermore, its principal strengths lie in confirmatory molecular weight assessment, the ability to detect protein isoforms and post-translational modifications, and low-cost, low-complexity implementation suited to small-scale validation studies. However, this method is comparatively less sensitive, lower-throughput, and less quantitative than mass spectrometry-based or immunoassay-based platforms when used for biomarker discovery or clinical deployment. In contrast, some techniques, such as liquid chromatography-tandem mass spectrometry/mass spectrometry, sequential window acquisition of all theoretical mass spectra data-independent acquisition-mass spectrometry, and ELISA or multiplex immunoassays, offer superior sensitivity, quantitative precision, and scalability, making them better suited for large-scale discovery, biomarker panel development, and eventual clinical translation (17-21).

It should be noted that TNF-α and IL-1β have well-documented, central roles in orthodontic tooth movement and root resorption triggered by orthodontic forces. In addition, saliva has growing promise as a non-invasive diagnostic medium. Accordingly, the current research aims to determine whether these two cytokines could be reliably measured in the saliva of patients undergoing orthodontic root resorption. It has been proposed that the increased salivary concentrations of TNF-α and IL-1β would be associated with the occurrence and extent of OIIRR. To test this claim, Western blot analysis was performed for its specificity and sensitivity in protein detection. More precisely, the goal was to assess the potential of IL-1β and TNF-α as non-invasive salivary biomarkers for the early detection and tracking of orthodontic root resorption, thereby contributing to safer orthodontic clinical practices. Moreover, Western blotting was selected as the primary analytical method because it allowed direct, specific confirmation of IL-1β and TNF-α at their expected molecular weights in a small, well-defined pilot cohort, providing a cost-effective and technically accessible means of establishing proof-of-concept evidence before committing to more resource-intensive, higher-throughput platforms for future validation.

## Materials and Methods

### *Study Design and Participants*

This study employed a case-control design, enrolling 24 individuals receiving orthodontic care. In addition, cases and controls were comparable in gender and age. Among them, 12 presented with radiographically confirmed external apical root resorption linked to orthodontic treatment (the case group), while the remaining 12 showed no radiographic indications of root resorption (the control group). Further, the average age for the case and control groups was 22.17 and 22.67, respectively. Furthermore, the average age of participants in the case group was 22.17 years compared with 22.67 years in the control group. Moreover, the gender distribution was 9 females to 2 males in the case group and 11 females to 1 male in the control group. Data related to individuals' age and gender are available in Supplementary Data 1. All patients underwent treatment using the McLaughlin, Bennett, Trevisi fixed appliance system (0.022-inch slot), following a uniform institutional protocol with comparable archwire sequences.

Likewise, participants were consecutively recruited at the Department of Orthodontics, Hamadan University of Medical Sciences, Hamadan, Iran, through a review of routine clinical records and radiographic images. Additionally, root resorption status was determined at a single time point based on radiographs taken either during active treatment (≥10 months into therapy) or upon treatment completion. Panoramic radiographs

were the primary diagnostic tool, and periapical views were used when additional detail was required. It should be noted that no radiographs were obtained exclusively for research purposes. An experienced orthodontist confirmed the diagnosis and classified resorption severity using Zary's grading system (grades I–IV). Only cases classified as grade II or higher were assigned to the case group. In addition, the group assignment was finalized before saliva collection.

Similarly, eligibility for both groups required extraction-based orthodontic therapy, a minimum of 24 natural teeth, and the absence of active cavitated caries. Informed written consent was also secured from every participant. The exclusion criteria included systemic illness, periodontal disease, smoking, a history of allergic reactions, antibiotic intake within three months before sampling, poor cooperation, and the use of complex orthodontic mechanics (e.g., intrusion arches, vertical elastics for open-bite correction, or high torque demands such as those observed in class II division 2 malocclusion). Moreover, baseline cephalometric parameters, specifically Sella-Nasion–mandibular plane and Frankfort–mandibular plane angle, measured before treatment initiation were required to fall within standard normative ranges (Steiner/Tweed/Proffit) in order to minimize confounding related to vertical skeletal pattern. Further, the total treatment duration was obtained from clinical records; the average treatment length was 39 months in the control group, with no patient having less than 36 months of active therapy.

### *Sample Collection and Processing*

Following informed consent, demographic and clinical data were documented using standardized questionnaires. To minimize circadian fluctuations, unstimulated whole saliva specimens were obtained between 9:00 and 11:00 in the morning (22-24). Furthermore, participants were instructed to avoid all food and beverage intake, as well as caffeine, alcohol, nicotine, chewing gum, and any oral hygiene measures, for at least 1 hour before sample collection (14, 23). After a deionized-water rinse and a 10-minute rest, saliva was collected by passive drool (14) into pre-chilled polypropylene tubes kept on ice; saliva from the first minute was discarded, and saliva collected during the subsequent 5 minutes was retained. Then, samples were transported on ice within 120 minutes (25), centrifuged at 12,000 × g for 15 minutes at 4°C (26), and 1 mL of the supernatant was supplemented with protease inhibitor cocktail (2 µL) and 1 mM sodium orthovanadate (3 µL; Sigma-Aldrich, St. Louis, MO, USA), aliquoted, and stored at −80°C until analysis (27). It is noteworthy that the specimens were collected at Hamadan University of Medical Sciences (2023–2025), and Western blot experiments were performed at Sorena Laboratory (Tehran, Iran).

### *Western Blotting*

Saliva specimens were thawed while kept on ice, and a protease inhibitor cocktail (Sigma–Aldrich, USA) was promptly added to each sample in order to prevent proteolysis. To eliminate cellular debris and mucin, the samples were spun at 14,000 × g for 15 minutes at 4°C. Subsequently, the clear supernatant was collected, and protein content was measured using the Bradford Protein Quantification kit (DB0017, DNAbioTech, Iran) according to the supplier's instructions. The supernatants were diluted 1:1 with 2X Laemmli sample buffer.

Aliquots containing 20 µg of protein were denatured at 95°C for 5 minutes and then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5% stacking and 10% separating gels). Next, they electroblotted onto 0.2 µm polyvinylidene difluoride membranes (Bio-Rad Laboratories, USA). Afterward, the membranes were blocked with 5% bovine serum albumin prepared in TBST (supplemented with 0.1% Tween-20) for 1 hour at room temperature. After blocking, the membranes were subjected to overnight incubation at 4°C with gentle agitation in the presence of primary antibodies directed against IL-1β (1:1000, Cat. No. ab254360, Abcam, USA), TNF-α (1:1000, Cat. No. ab307164, Abcam, USA), and β-actin, used as a loading control (1:2500, Cat. No. ab8227, Abcam). Following three TBST washes, the membranes were incubated with a goat anti-rabbit immunoglobulin G H&L (horseradish peroxidase) secondary antibody (1:10000, Cat. No. ab6721, Abcam) for 1 hour at ambient temperature.

Moreover, enhanced chemiluminescence reagents were used for protein band detection. Additionally, densitometry was performed using GelAnalyzer (version 2010a, NIH, USA), with target protein levels normalized to β-actin. Normalized values were then compared between groups as described above (28). Therefore, the protein expression was expressed as the ratio of target protein signal to β-actin, yielding a dimensionless relative expression value. These values are reported as arbitrary units, as no absolute mass or molar quantification was performed.

### *Statistical Analyses*

Data normality was checked with the Shapiro–Wilk test. Group comparisons employed independent-samples t-tests (equal variances assumed), and effect sizes were estimated using Cohen's d (small: 0.2, medium: 0.5, large: ≥ 0.8). Furthermore, receiver operating characteristic (ROC) curves were constructed to evaluate diagnostic accuracy, with optimal cutoffs determined by the Youden index (J = sensitivity + specificity − 1). Finally, the area under the curve (AUC) values were obtained using R software (version 4.4.1).

## Results

### *Elevated Expression Levels of Interleukin-1β and Tumor Necrosis Factor-α in the Case Group*

Western blotting was utilized to examine the salivary protein levels of TNF-α and IL-1β, with values normalized to β-actin. The results revealed a marked increase in both markers within the case group when compared with

healthy controls (Figure 1).

Based on the results (Figure 2), the mean relative expression of IL-1β was substantially higher in the case group (1.36 ± 0.33) than in the control group (0.68 ± 0.16). An independent-samples t-test confirmed that this difference was statistically significant ($t(22) = 6.32$, $P < 0.0001$). Moreover, the calculated effect size was large, with Cohen's $d = 2.58$ (95% confidence interval [CI]: 1.44–3.73). Likewise, TNF-α expression was substantially higher in the case group (1.38 ± 0.29) than in the control group (0.80 ± 0.15). This difference also reached statistical significance ($t(22) = 6.27$, $P < 0.0001$), and a large effect size was observed (Cohen's $d = 2.56$, 95% CI: 1.42–3.70).

### *Diagnostic Accuracy of Tumor Necrosis Factor-α and Interleukin-1β*

ROC curves were generated to assess the usefulness of IL-1β and TNF-α as diagnostic biomarkers. Both molecules displayed outstanding diagnostic performance. For IL-1β, the AUC was 0.986 (Figure 3a). Using the Youden index (J = 0.917), the optimal cutoff for separating case subjects from controls was 0.875. In addition, the ROC analysis revealed an AUC of 0.951 for TNF-α (Figure 3b). The optimal threshold was set at 1.1, yielding a Youden index of 0.917.

## Discussion

OIIRR is a common adverse effect that results from orthodontic treatment, often progressing without clinical signs until substantial damage has occurred. Non-invasive biomarkers hold promise for the early identification and longitudinal surveillance of this condition (29). This study assessed the salivary levels of two key cytokines (IL-1β and TNF-α) in patients with OIIRR compared with orthodontic individuals without root resorption.

The available evidence positions IL-1β and TNF-α as central, mechanistically plausible drivers of inflammatory root resorption, with demonstrable effects confirmed by experimental modulation. Absolute effect estimates derived from animal intervention and pulpectomy studies suggest that interrupting cytokine signaling or eliminating the intraradicular sources of these mediators can yield clinically meaningful reductions in resorption. However, several gaps hinder the translation of these findings to human practice remains. Candidate gene association studies hint at the potential for patient stratification but require larger, ethnically diverse cohorts and genome-wide approaches in order to move beyond modest relative risks and mitigate publication bias. Likewise, interventional strategies specifically designed to antagonize IL-1β or TNF-α at the periodontal root apex have yet to be tested in randomized clinical trials, and systemic cytokine blockade would carry significant off-target risks. Local modulation through biomaterials or targeted delivery of anti-inflammatory agents offers a plausible translational pathway, which is supported by biomaterial polarization data. Nevertheless, it requires controlled studies assessing preserved tooth movement, infection risk, and long-term root integrity. Accordingly, future work should quantify dose-response relationships between force magnitude and cytokine induction using standardized metrics, integrate the single-cell resolution of pulp and periodontal cell contributions, and pursue randomized controlled biomaterial or local pharmacologic interventions with predefined imaging-based resorption endpoints. Until then, the convergence of mechanistic and animal evidence supports a model in which IL-1β and TNF-α amplify odontoclastic activity and suggests that the modulation of these cytokines, or their upstream triggers, can substantially reduce resorption burden while preserving orthodontic efficacy (30-34).

Ciurla et al (35) explored whether single-nucleotide polymorphisms (SNPs) in IL-1β, TNF receptor superfamily member 11B (TNFRSF11B), caspase 1 (CASP1), and IL-6 are linked to external apical root resorption (EARR) development during orthodontic treatment. Radiographic images taken before and after therapy from 101 patients underwent examination. The results revealed a notable association between EARR and the *IL-1β* gene variant, which increased the risk of EARR progression by roughly

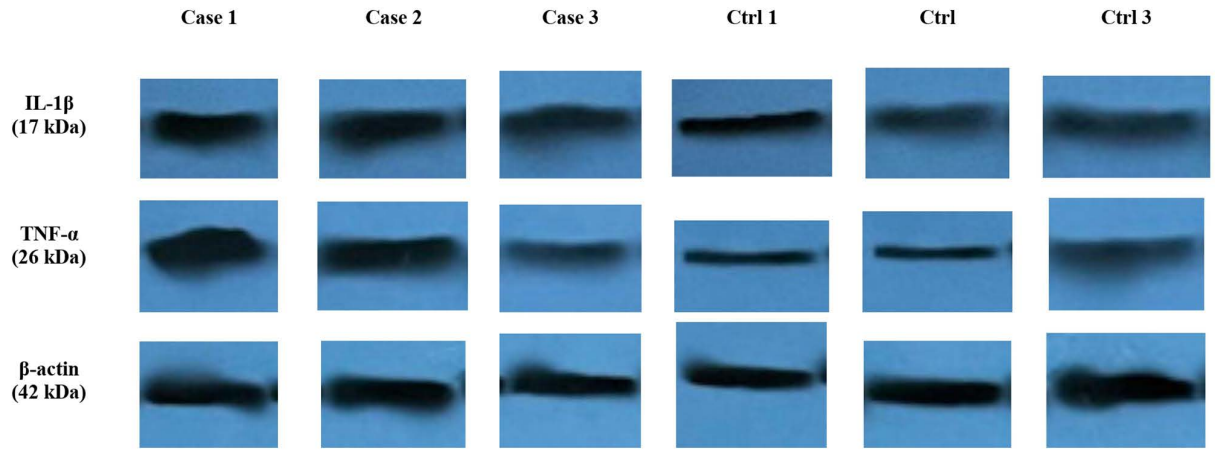



**Figure 1.** Representative Western Blot Images of Salivary IL-1β, TNF-α, and β-Actin in OIIRR Cases and Controls
*Note*. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; OIIRR: Orthodontically induced inflammatory root resorption. Cropped immunoblots show IL-1β (17 kDa), TNF-α (26 kDa), and β-actin (42 kDa) bands from three representative OIIRR patients (cases 1–3) and three controls (Ctrl 1–3)

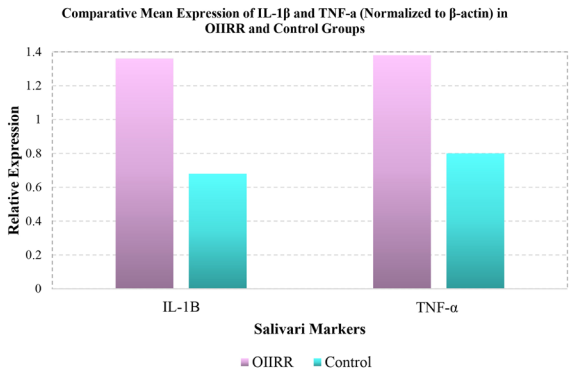



**Figure 2.** Quantitative Comparison of IL-1β and TNF-α Protein Expression (Relative to β-Actin) in OIIRR Patients and Healthy Controls
*Note*. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; OIIRR: Orthodontically induced inflammatory root resorption; SD: Standard deviation. Bar graphs depict significantly elevated salivary IL-1β levels in OIIRR cases (1.36 ± 0.33) compared with controls (0.68 ± 0.16; $P < 0.0001$) and similarly increased TNF-α levels in cases (1.38 ± 0.29) versus controls (0.80 ± 0.15; $P < 0.0001$). Data are shown as mean relative expression ± SD (purple bars: OIIRR, n = 12; cyan bars: Controls, n = 12)

4 times. In contrast, no meaningful links were detected between EARR and SNPs in TNFRSF11B, CASP1, or IL6. While Ciurla et al (35) linked an IL-1β SNP to a 4-fold higher EARR risk, the protein-level analysis in our study demonstrated significantly elevated salivary IL-1β in cases versus controls. This aligns with their genetic findings, suggesting that the risk variant may increase IL-1β expression.

Bastos Lages et al (36) examined whether an *IL-1β* gene variant is related to EARR during orthodontic therapy. Among 61 Brazilian orthodontic patients, 23 had EARR and 38 served as controls. DNA from buccal swabs was genotyped, revealing marked differences in the allele and genotype distributions of the IL-1β polymorphism between the groups. Allele 1 was associated with a fourfold increased risk of EARR (odds ratio = 4.0). The scholars concluded that this IL-1β polymorphism is substantially associated with root resorption in the studied population. Although genotyping was not performed in the present

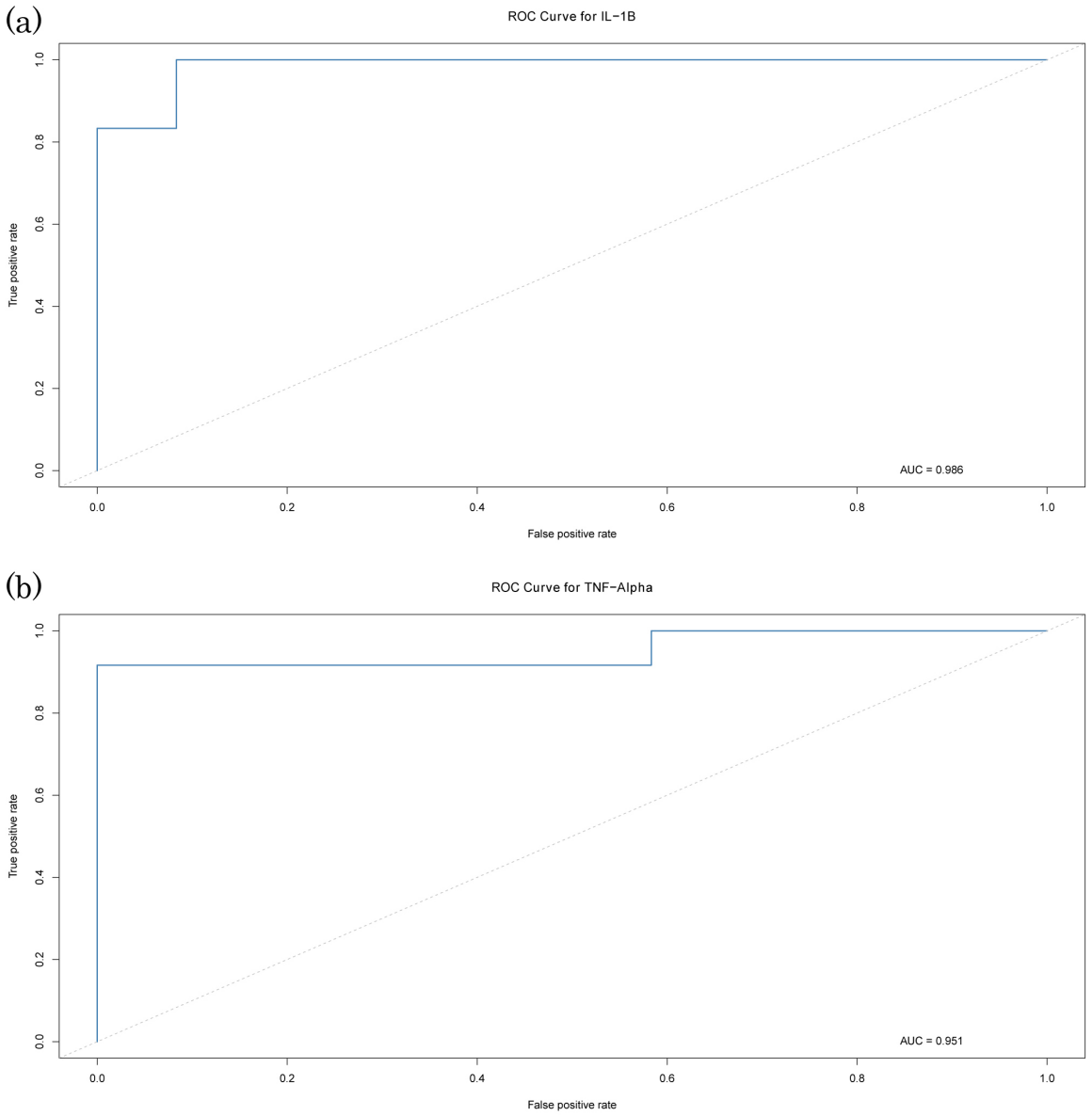



**Figure 3.** Area Under the Curve Derived From Western Blot Analyses of **(a)** IL-1β and **(b)** TNF-α
*Note*. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; AUC: Area under the curve; ROC: Receiver operating characteristic

study, the markedly elevated salivary IL-1β protein levels observed in the case group are consistent with the genetic susceptibility described by Bastos Lages et al (36), further supporting IL-1β's role as a biomarker of root resorption risk.

Zhang et al (37) investigated how the nucleotide-binding domain, leucine-rich–containing family, pyrin domain containing 3 (NLRP3) inflammasome and M1 macrophages contribute to root resorption. Using a rat model of root resorption caused by excessive orthodontic force, they found that NLRP3, caspase-1, IL-1β, and M1 macrophages were all highly concentrated around the areas of root resorption. In a cell co-culture system, they further showed that force-treated human periodontal ligament cells promoted M1 macrophage polarization and increased the production of NLRP3, caspase-1, and IL-1β. In addition, both NLRP3 activation and M1 macrophage polarization were blocked when they added an NLRP3 inhibitor (MCC950). The researchers concluded that force-stimulated periodontal ligament cells activate the NLRP3 inflammasome in M1 macrophages, leading to increased IL-1β production and the onset of inflammatory root resorption. In our study, the elevated salivary IL-1β levels in the case group may be biologically explained by this mechanistic pathway, indicating that the NLRP3/IL-1β axis activated by orthodontic force in animal models could be captured as a non-invasive biomarker signal in humans with OIIRR.

Similarly, Tang et al (38) attempted to understanding how the mechanosensitive molecule alpha epithelial sodium channel (αENaC) contributes to external root resorption (ERR) of the mandibular second molar associated with a fully impacted third molar. Their findings confirmed that mechanical pressure from the impacted tooth was concentrated in the resorption zone, and αENaC expression was markedly increased in the adjacent granulation tissues. Additionally, they observed a direct correlation between αENaC levels and both the extent of ERR and the concentrations of TNF-α and N-cadherin, suggesting a connection among mechanical loading, inflammatory processes, and root resorption. It should be noted that the positive correlation between αENaC, TNF-α, and resorption extent parallels the significantly higher salivary TNF-α levels observed in our OIIRR cases relative to controls, further supporting TNF-α as a mechanosensitive inflammatory mediator of root resorption that can be detected non-invasively in saliva.

Likewise, Lin et al (39) conducted a review to elucidate the pathophysiological mechanisms underlying non-infectious inflammatory root resorption in permanent teeth. According to their analysis, tissue injury (e.g., from excessive orthodontic forces) induces hypoxic conditions and triggers the release of damage-associated molecular patterns (DAMPs). This sterile injury triggers an immune response in which macrophages and neutrophils secrete crucial inflammatory mediators, particularly TNF-α and IL-1. In turn, these cytokines activate macrophage cells in order to remove injured tissue and bone during wound healing. It I worth mentioning that this process may inadvertently result in root resorption when the protective precementum and cementoblast layers are disrupted. The elevated salivary IL-1β and TNF-α in our OIIRR cases align with the DAMP-driven inflammatory cascade, highlighting their role as non-invasive biomarkers of immune-mediated resorption as described by Lin et al (39).

These findings establish IL-1β and TNF-α as convergent biomarkers of OIIRR across genetic, mechanistic, and immunological domains. Genetic evidence from Ciurla et al (35) and Bastos Lages et al (36) associates IL-1β polymorphisms with heightened resorption risk; mechanistic studies by Zhang et al (37) and Tang et al (38) demonstrated that mechanical loading activates NLRP3/IL-1β signaling and induces αENaC-dependent TNF-α release in the periodontal ligament. Moreover, the work of Lin et al (39) delineated a DAMP-triggered macrophage cascade through which sterile inflammatory stimuli propagate root resorption. Furthermore, our observation of substantially elevated salivary IL-1β and TNF-α in OIIRR cases, together with strong diagnostic performance, bridges these genetic and animal-model insights with a clinically accessible, non-invasive biomarker. This convergence supports a unified framework in which force-induced IL-1β/TNF-α signaling underpins root resorption and is reflected in the salivary proteome.

In this study, several constraints merit consideration. The limited sample size restricted generalizability, and findings require replication in larger cohorts. Western blotting, while specific, is semi-quantitative, and absolute methods (e.g., ELISA) may offer greater precision. In addition, panoramic radiography was the primary diagnostic tool, though cone-beam computed tomography would allow more accurate three-dimensional evaluation. Further, confounders such as force levels, treatment duration, and genetic factors were not completely controlled. Additionally, body mass index and obesity-related inflammatory status were not evaluated in this study; therefore, the potential role of obesity in influencing OIIRR risk or salivary cytokine levels remains unclear, warranting investigation in future studies. Likewise, excellent diagnostic performance was demonstrated for IL-1β and TNF-α; however, these estimates were obtained from the same small cohort used for ROC curve generation, which may introduce optimism bias. The reported diagnostic utility, optimal cutoffs, and Youden indices should, therefore, be regarded as preliminary, thereby requiring validation in an independent group of OIIRR patients and controls before they can be considered for clinical use. Given that the case group included only patients with class II or higher resorption, the reported diagnostic performance reflected the detection of moderate-to-severe OIIRR rather than early-stage disease. As cytokine levels may correlate with severity, the cutoffs' sensitivity for mild (class I) resorption is uncertain. Hence, future studies covering all severity grades are needed to assess whether

these biomarkers support early diagnosis, not just the confirmation of advanced OIIRR.

## Conclusion

It was revealed that salivary IL-1β and TNF-α are significantly upregulated in orthodontic patients with confirmed root resorption compared with those without. Both markers demonstrated large effect sizes and exceptional diagnostic accuracy, with AUC values above 0.95. Therefore, these results support the potential of these cytokines as non-invasive salivary biomarkers for early detection and monitoring of OIIRR. Pending validation through larger prospective studies, these biomarkers could aid in risk stratification, enable timely intervention, and advance more individualized orthodontic treatment approaches.

### Acknowledgments

The authors would like to thank Hamadan University of Medical Sciences, Hamadan, Iran, and Dr. Vahid Mollabashi for their support.

### Artificial Intelligance Use Disclosure

GenAI tools were used to improve spelling, grammar, general editing, and writing style. Subsequently, the manuscript underwent grammatical editing with Grammarly Premium. Moreover, all authors read and approved the final manuscript.

### Authors' Contribution

Conceptualization: Amir Taherkhani, Homa Farhadifard
Data Curation: Parnia Yari, Parzhin Moradi
Formal Analysis: Amir Taherkhani, Vahid Siavashi
Investigation: Amir Taherkhani, Homa Farhadifard, Parzhin Moradi
Methodology: Amir Taherkhani, Vahid Siavashi
Project Administration: Amir Taherkhani, Homa Farhadifard
Resources: Amir Taherkhani
Software: Amir Taherkhani, Vahid Siavashi
Supervision: Amir Taherkhani, Homa Farhadifard
Validation: Amir Taherkhani, Vahid Siavashi, Homa Farhadifard
Visualization: Amir Taherkhani, Vahid Siavashi
Writing–Original Draft: Amir Taherkhani, Vahid Siavashi
Writing–Review & Editing: Homa Farhadifard

### Competing Interests

The authors declare that they have no competing interests.

### Consent to Publish Declaration

Not applicable.

### Data Availability Statement

Due to privacy/ethical restrictions, data related to this study are available upon reasonable request.

### Ethical Approval

The present study was approved by the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (ethics No. IR.UMSHA.REC.1405.045; Project No. 14050201702). In addition, the study adhered to the Declaration of Helsinki (2013).

### Funding

This research received a specific grant from Hamadan University of Medical Sciences, Hamadan, Iran (grant No. 48145).

### Informed Consent

Written informed consent forms were obtained from all participants for the use of biological samples.

### Supplementary File

**Supplementary Data 1.** A total of 24 volunteers, including 12 cases and 12 controls, contributed to this study.

### References

1. Li B, Wang L, He H. Autophagy in orthodontic tooth movement: advances, challenges, and future perspectives. Mol Med 2025;31(1):245. doi:10.1186/s10020-025-01299-y
2. Sondeijker CF, Lamberts AA, Beckmann SH, Kuitert RB, van Westing K, Persoon S, et al. Development of a clinical practice guideline for orthodontically induced external apical root resorption. Eur J Orthod 2020;42(2):115-24. doi:10.1093/ejo/cjz034
3. Krishnan V, Davidovitch Z. Cellular, molecular, and tissue-level reactions to orthodontic force. Am J Orthod Dentofacial Orthop 2006;129(4):469.e1-32. doi:10.1016/j.ajodo.2005.10.007
4. Meikle MC. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. Eur J Orthod 2006;28(3):221-40. doi:10.1093/ejo/cjl001
5. Putranto R, Oba Y, Kaneko K, Shioyasono A, Moriyama K. Effects of bisphosphonates on root resorption and cytokine expression during experimental tooth movement in rats. Orthod Waves 2008;67(4):141-9. doi:10.1016/j.odw.2008.04.002
6. Liu C, Chu D, Kalantar-Zadeh K, George J, Young HA, Liu G. Cytokines: from clinical significance to quantification. Adv Sci (Weinh) 2021;8(15): e2004433. doi:10.1002/advs.202004433
7. Borish LC, Steinke JW. 2. Cytokines and chemokines. J Allergy Clin Immunol 2003;111(2 Suppl): S460-75. doi:10.1067/mai.2003.108
8. Garlet TP, Coelho U, Silva JS, Garlet GP. Cytokine expression pattern in compression and tension sides of the periodontal ligament during orthodontic tooth movement in humans. Eur J Oral Sci 2007;115(5):355-62. doi:10.1111/j.1600-0722.2007.00469.x
9. Yamaguchi M. RANK/RANKL/OPG during orthodontic tooth movement. Orthod Craniofac Res 2009;12(2):113-9. doi:10.1111/j.1601-6343.2009.01444.x
10. Curl L, Sampson W. The presence of TNF-alpha and TNFR1 in aseptic root resorption. A preliminary study. Aust Orthod J 2011;27(2):102-9.
11. Li Y, Ou Y, Fan K, Liu G. Salivary diagnostics: opportunities and challenges. Theranostics 2024;14(18):6969-90. doi:10.7150/thno.100600
12. Song M, Bai H, Zhang P, Zhou X, Ying B. Promising applications of human-derived saliva biomarker testing in clinical diagnostics. Int J Oral Sci 2023;15(1):2. doi:10.1038/s41368-022-00209-w
13. Kumari S, Samara M, Ampadi Ramachandran R, Gosh S, George H, Wang R, et al. A review on saliva-based health diagnostics: biomarker selection and future directions. Biomed Mater Devices 2024;2(1):121-38. doi:10.1007/s44174-023-00090-z
14. Xu S, Mumuni AN, Tuason RT, Maki KA. Methodological considerations in saliva-based biomarker research: addressing patient-specific variability in translational research protocols. Curr Protoc 2025;5(10): e70235. doi:10.1002/cpz1.70235
15. Zubair M, Launico MV. Western blot: principles, procedures, and clinical applications. In: StatPearls [Internet]. Treasure Island, FL: StatPearls Publishing; 2025.
16. Torian LV, Forgione LA, Punsalang AE, Pirillo RE, Oleszko WR. Comparison of Multispot EIA with Western blot for confirmatory serodiagnosis of HIV. J Clin Virol 2011;52 Suppl 1: S41-4. doi:10.1016/j.jcv.2011.09.017
17. Trevisan França de Lima L, Müller Bark J, Rasheduzzaman M, Weeramange CE, Punyadeera C. Isolation and characterization

of salivary exosomes for cancer biomarker discovery. Methods Mol Biol 2022;2504:101-12. doi:10.1007/978-1-0716-2341-1_8
18. Rao PV, Reddy AP, Lu X, Dasari S, Krishnaprasad A, Biggs E, et al. Proteomic identification of salivary biomarkers of type-2 diabetes. J Proteome Res 2009;8(1):239-45. doi:10.1021/pr8003776
19. Ploypetch S, Roytrakul S, Suriyaphol G. Salivary proteomic analysis of canine oral melanoma by MALDI-TOF mass spectrometry and LC-mass spectrometry/mass spectrometry. Methods Mol Biol 2021;2265:429-45. doi:10.1007/978-1-0716-1205-7_31
20. Zhang X, Walsh T, Atherton JJ, Kostner K, Schulz B, Punyadeera C. Identification and validation of a salivary protein panel to detect heart failure early. Theranostics 2017;7(18):4350-8. doi:10.7150/thno.21727
21. Esteves CV, de Campos WG, Amorim Dos Santos J, Kobayashi Velasco S, Guerra ENS, Siqueira WL, et al. Proteomic profile of saliva collected directly from ducts: a systematic review. Clin Oral Investig 2020;24(2):559-68. doi:10.1007/s00784-019-03165-8
22. Ostheim P, Alemu SW, Tichý A, Sirak I, Davidkova M, Stastna MM, et al. Examining potential confounding factors in gene expression analysis of human saliva and identifying potential housekeeping genes. Sci Rep 2022;12(1):2312. doi:10.1038/s41598-022-05670-5
23. Parihar AS, Tariq M, Singh AK, Mathur P, Bhattacharjee A, Priyadarshini K. Salivary biomarkers vs. conventional biopsy for oral cancer detection in India: a comparative study. J Pharm Bioallied Sci 2025;17(Suppl 1): S278-80. doi:10.4103/jpbs.jpbs_152_25
24. Khoo SC, Shoji Y, Teh CH, Ma NL. Discovery of biomarkers for myogenous temporomandibular disorders through salivary metabolomic profiling: a pilot study. J Oral Facial Pain Headache 2023;37(3):207-16. doi:10.11607/ofph.3353
25. Subramaniam P, Kumar K, Girish Babu KL. Levels of salivary immunoglobulin A (SIgA) in HIV infected children. J Clin Pediatr Dent 2015;39(4):377-81. doi:10.17796/1053-4628-39.4.377
26. Nigar S, Hassan S, Maqsood A, Ahmed N, Al-Askar M, Mokeem SA, et al. An assessment of unstimulated salivary flow rate, IgA and clinical oral dryness among active and passive smokers. Int J Occup Med Environ Health 2022;35(1):39-51. doi:10.13075/ijomeh.1896.01829
27. Ordonez EB, Sprave T, Thomsen AR, Schäfer H, Grosu AL, Jendrossek V, et al. Increased acid sphingomyelinase levels in saliva as oral mucositis severity predictors. Front Oncol 2025;15:1613884. doi:10.3389/fonc.2025.1613884
28. Asadian S, Alibabrdel M, Daei N, Cheraghi H, Maedeh Jafari S, Noshadirad E, et al. Improved angiogenic activity of endothelial progenitor cell in diabetic patients treated with insulin plus metformin. J Cell Biochem 2019;120(5):7115-24. doi:10.1002/jcb.27985
29. Ghani AA, Rashid ME, Alhodhudi A, Ibrahim T, Hajeer MY, Alam MK. Assessment of salivary exosomal microRNAs as biomarkers for orthodontically induced root resorption: a prospective study. J Pharm Bioallied Sci 2025;17(Suppl 4): S3283-5. doi:10.4103/jpbs.jpbs_1350_25
30. Nieto-Nieto N, Solano JE, Yañez-Vico R. External apical root resorption concurrent with orthodontic forces: the genetic influence. Acta Odontol Scand 2017;75(4):280-7. doi:10.1080/00016357.2017.1294260
31. Lara VS, Figueiredo F, da Silva TA, Cunha FQ. Dentin-induced in vivo inflammatory response and in vitro activation of murine macrophages. J Dent Res 2003;82(6):460-5. doi:10.1177/154405910308200611
32. Tu MG, Sun KT, Wang TH, He YZ, Hsia SM, Tsai BH, et al. Effects of mineral trioxide aggregate and bioceramics on macrophage differentiation and polarization in vitro. J Formos Med Assoc 2019;118(10):1458-65. doi:10.1016/j.jfma.2019.07.010
33. Kaku M, Yamamoto T, Yashima Y, Izumino J, Kagawa H, Ikeda K, et al. Acetaminophen reduces apical root resorption during orthodontic tooth movement in rats. Arch Oral Biol 2019;102:83-92. doi:10.1016/j.archoralbio.2019.04.002
34. Kaku M, Sumi H, Shikata H, Kojima S, Motokawa M, Fujita T, et al. Effects of pulpectomy on the amount of root resorption during orthodontic tooth movement. J Endod 2014;40(3):372-8. doi:10.1016/j.joen.2013.11.027
35. Ciurla A, Marruganti C, Doldo T, Szymańska J. Association between polymorphisms in the IL-1β, TNFRSF11B, CASP1, and IL-6 genes and orthodontic-induced external apical root resorption. J Clin Med 2021;10(18):4166. doi:10.3390/jcm10184166
36. Bastos Lages EM, Drummond AF, Pretti H, Costa FO, Lages EJ, Gontijo AI, et al. Association of functional gene polymorphism IL-1beta in patients with external apical root resorption. Am J Orthod Dentofacial Orthop 2009;136(4):542-6. doi:10.1016/j.ajodo.2007.10.051
37. Zhang J, Liu X, Wan C, Liu Y, Wang Y, Meng C, et al. NLRP3 inflammasome mediates M1 macrophage polarization and IL-1β production in inflammatory root resorption. J Clin Periodontol 2020;47(4):451-60. doi:10.1111/jcpe.13258
38. Tang J, Yu W, Lin L, Yang R, Li G, Jin M, et al. Role of αENaC in root resorption of adjacent teeth due to entirely impacted mandibular third molars. BMC Oral Health 2024;24(1):360. doi:10.1186/s12903-024-04040-z
39. Lin S, Marvidou AM, Novak R, Moreinos D, Abbott PV, Rotstein I. Pathogenesis of non-infection related inflammatory root resorption in permanent teeth: a narrative review. Int Endod J 2023;56(12):1432-45. doi:10.1111/iej.13976