Original Article

Evaluation of IL-1β and CRP mRNA Expression Levels by RT-PCR in Postorthodontic Treatment Patients with Temporomandibular Joint Disorders: A Cross-Sectional Study

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Received : 08-Dec-2023
Revised : 10-Jan-2024
Accepted : 19-Jan-2024
Published: 29-Apr-2024

ABSTRACT

Aim: Temporomandibular joint disorder (TMD), which affects the masticatory muscles, temporomandibular joint, and surrounding tissues, can manifest as inflammation. This study aims to explore the expression levels of the inflammatory biomarkers, interleukin (IL)-1β and C-reactive protein (CRP), in TMD patients who have undergone orthodontic treatment. **Materials and Methods:** Buccal swabs from 105 postorthodontic treatment patients were analyzed using real-time polymerase chain reaction to assess the expression levels of IL-1β and CRP in each group after messenger ribonucleic acid extraction. Patients were also examined using the Diagnostic Criteria for TMD (DC/TMD) to determine if they met the criteria for a TMD diagnosis. The TMD group was subdivided into three categories based on the DC/TMD. **Results:** The study included 37 patients who did not develop TMD (group 0) and 68 participants who developed TMD after orthodontic treatment, including 17 with pain-related TMDs (group 1), 29 with intra-articular TMDs (Group 2), and 22 with combined pain-related and intra-articular TMDs (group 3). CRP expression was higher than IL-1β in groups 1 and 2, and IL-1β expression was higher than CRP in group 3. The Kruskal–Wallis test showed that IL-1β and CRP expression levels in groups 1, 2, and 3 were not statistically different. Sex and adult age had considerable effects on the occurrence of TMD in patients after orthodontic treatment. **Conclusions:** Higher IL-1β expression was found in postorthodontic treatment patients with more complex TMD. This study strengthens the evidence of inflammation through IL-1β and CRP expression in individuals with TMD, especially after orthodontic treatment

Keywords: *C-reactive protein, inflammatory cytokines, interleukin-1β, orthodontic treatment, real-time polymerase chain reaction, temporomandibular joint disorders*

Introduction

Temporomandibular joint disorder (TMD) is a collection of symptoms with various clinical manifestations involving masticatory muscles, temporomandibular joint (TMJ), mandibular movements, and surrounding structures.[1-4] The prevalence of TMD in the form of pain and other functional disorders ranges from 10% to 30%. TMD is prevalent among women aged 20–40 years. However,

Access this article online

Quick Response Code:

Website: https://journals.lww.com/jpcd

DOI: 10.4103/jispcd.jispcd_197_23

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How to cite this article: Ismah N, Bachtiar EW, Purwanegara MK, Tanti I, Mardiati E. Evaluation of IL-1β and CRP mRNA expression levels by RT-PCR in postorthodontic treatment patients with temporomandibular joint disorders: a cross-sectional Study. J Int Soc Prevent Communit Dent 2024;14:98-104.

this does not preclude the likelihood that it could also occur in children.[2,5-7]

The etiology of TMD is likely multifactorial. The leading cause of TMD is believed to be occlusal factors, with emotional stress factors also contributing.[8] Occlusion has been associated with the development of TMD.[9] Numerous studies have examined the correlation between occlusive factors and the occurrence of TMD; however, the conclusions remain unclear.[6,10] Patients with abnormal occlusion accompanied by TMD have shown improvement after orthodontic treatment, with some experiencing complete resolution of their TMD after treatment. Research on this subject is still ongoing.[11]

Like other inflammatory diseases, TMD may be associated with increased levels of specific biomarkers, indicating the occurrence of pathogenic processes.[4,12] Mechanical nociceptor stimulation leads to elevated levels of neuropeptides, inflammatory mediators, and local hypoxia. Several biomarkers, including serum macrophage chemotactic factor-1, macrophage inflammatory protein-1 alpha, and serum C-reactive protein (CRP), may indicate inflammation caused by musculoskeletal issues and provide insights into the extent of inflammation associated with these issues.[13]

The inflammatory pathophysiology of TMD involves the release of the cytokines interleukin (IL)-1β and CRP, which contribute to the body's defense reaction. Common biomarkers present in TMD include IL-6, IL-1, IL-8, tumor necrosis factor (TNF), and CRP.[4,13] IL-1 is an important physiological cytokine that promotes inflammation and amplifies the immune response. Activation of the inflammatory cytokine IL-1β is triggered via the IL 1 receptor 1 (IL-1R1), which is the first receptor to bind with IL-1α and IL-1β agonist ligands, leading to responses by a wide variety of cell types, including T cells, fibroblasts, endothelial cells, and epithelial cells.[12] CRP is a reliable biomarker of acute reactions to inflammatory and infectious conditions.[4,14] The appearance of CRP can additionally identify bone injury in the TMJ via proteinases and cytokines (TNF-α, IL-1β, and IL-6), indicating inflammation.[14,15]

The most common type of TMD identified after orthodontic treatment remains uncertain. Furthermore, no association has been observed between orthodontic treatment and TMD.[16] The expression patterns of IL-1β and CRP in posttreatment orthodontic patients with different types of TMD are also unknown. Generally, TNF, IL-8, IL-6, and IL-1β are among several biomarkers associated with TMD.[17] However, the level of IL-1β and CRP expression in patients who have undergone orthodontic treatment and developed different types of TMD is unknown. Therefore, this study aimed to evaluate IL-1β and CRP expression levels in postorthodontic treatment patients who developed TMD.

Materials and Methods

The study was approved by the Dental Ethics Committee of the Faculty of Dentistry at Universitas Indonesia, Jakarta, Indonesia, under reference number 11/Ethical Approval/FKGUI/III/2022, with protocol number 070080222. All procedures were performed as per the ethical guidelines laid down by the Declaration of Helsinki. This research was conducted at the Dental and Oral Hospital, Orthodontic Unit, Faculty of Dentistry, Universitas Indonesia. The study took place between November 2022 and July 2023 and conformed to the Strengthening the Reporting of Observational Studies in Epidemiology guidelines.[18] Patients were recruited using convenience sampling, as this method is considered suitable for detecting TMD cases, where the prevalence is only 5%–10%, as it is cheap, efficient, quick, and easy.[2,19] One hundred and five postorthodontic treatment patients met the inclusion criteria and agreed to participate in the study after providing informed consent. The inclusion criteria for participants were patients who had completed orthodontic treatment within the past year and were using a retainer. The participants also had to have good general health, good oral hygiene, and not be taking any medications. The exclusion criteria were postoperative orthognathic patients, those with a history of craniofacial trauma, and those receiving treatment for TMD. All patients provided demographic data and underwent Diagnostic Criteria for Temporomandibular Disorders (DC/TMD) examinations for placement in groups. There were four groups: non-TMD (group 0), pain-related TMDs (group 1), intra-articular TMDs (group 2), and combined pain-related and intra-articular TMDs (group 3).[20]

Collecting the buccal swab

Following the clinical examination, each participant underwent a buccal swab procedure using a soft, nylon, bristle cytology brush. The buccal swab is a noninvasive sampling method, easy to collect, cost-effective, and provides good-quality results for wordy research applications, such as deoxyribonucleic acid (DNA) testing disease, forensics, and genealogy.[21,22] The samples were stored in microtubes (Eppendorf, Nest, China) containing phosphate-buffered saline and were held at −20°C for subsequent DNA extraction.[23]

Messenger Ribonucleic Acid Extraction And Transcription

Ribonucleic acid (RNA) was extracted by rotating an Eppendorf tube with nylon brushes for 30 s, followed

by centrifugation at 300 × *g* for 5 min. The nylon brush was removed near the flame, and the liquid in the Eppendorf tube was centrifuged again at 300 × *g* for 5 min to separate the pellet. The clear liquid at the top was removed, leaving the sediment (cell pellets) ready for extraction. The separation stage for RNA extraction followed the recommended procedure of adding 200 µL of chloroform per 1 mL of GENEzol™ Reagent (Reagent Geneaid Biotech Ltd., New Taipei City, Taiwan), which resulted in three layers, a clear layer at the top (RNA), a white layer/border in the middle, and a red layer at the bottom. The upper layer, which contained the RNA, was transferred to a new Eppendorf tube. The next stage was an RNA deposition stage using isopropanol, then an RNA washing stage using 70% ethanol, and finally an RNA resuspension stage using RNase-free water, after which samples were stored at −70°C.[24]

The concentrations of extracted RNA were measured using a NanoDrop2000™ spectrophotometer machine (Thermo Fisher Scientific, Waltham, MA, USA). The concentrations were equalized to 100 µUnits by adding RNase-free water.[23] The messenger ribonucleic acid (mRNA) samples were then transcribed into complementary DNA (cDNA) using the reverse transcript enzyme ReverTra-Ace® (Toyobo Inc., Osaka, Japan). The cDNA obtained was amplified in duplicate on an ABI StepOnePlus Real-Time PCR system using a SensiFAST SYBR Hi-ROX Mix. All procedures were performed according to the manufacturer's instructions. We utilized glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IL-1β, and CRP, complete with sequences GAPDH F: 5′-AATGGAAATCCCATCACCATCT-3′, R: 5′-CAGCATCGCCCCACTTG-3′, IL-1β F: 5′-ACGATGCACCTGTACGATCA-3′, R: 5′-TCTTTCAACACGCAGGACAG-3′, and CRP F: 5′-TGGCCAGACAGACATGTCGAGG-3′, R: 5′-AGTGGAGGCACACAGTGAAGGC-3′.[25,26]

The polymerase chain reaction (PCR) conditions were established as follows:[25]

Stage	Condition
Pre-denaturation	95°C for 5 min
Annealing and final extension	40 cycles of 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 5 min
Melting curve	95°C for 15 s, 60°C for 60 s, and 95°C for 15 s

Statistical analysis

Statistical analysis of IL-1β and CRP results investigated differences in $2^{-\Delta\Delta Ct}$ values and is presented in univariate and bivariate forms. The correlation between participant characteristics was also explored using the SPSS Statistics software for Windows version 29 (IBM, Armonk, NY, USA).

Results

One hundred and five buccal swabs were collected from patients who had undergone orthodontic treatment and fulfilled the inclusion criteria. This group was comprised of 77 women (73.3%) and 28 men (26.7%), with an average age of 26.4 years. Most patients had a class 1 malocclusion (55.2%). There were 37 non-TMD participants (group 0) and 68 who developed TMD. The TMD group was further subdivided into 17 participants with pain-related TMDs (group 1), 29 with intra-articular TMDs (group 2), and 22 with combined pain-related and intra-articular TMDs (group 3).

Shapiro–Wilk's test results from reverse transcription-polymerase chain reaction (RT-PCR) for $2^{-\Delta\Delta Ct}$ IL-1β and CRP values indicated an abnormal data distribution. The mean IL-1β value was 1.81 ± 5.04, and the mean CRP value was 2.41 ± 4.02 in participants with TMD [Figure 1].

IL-1β expression was highest in group 3 and lowest in group 1. CRP expression was highest in group 2 and lowest in group 3. CRP expression was more elevated than IL-1β in groups 1 and 2; however, IL-1β expression was higher than CRP in group 3 [Figure 2]. The Kruskal–Wallis test showed a $P > 0.05$, demonstrating no statistically significant differences in IL-1β and CRP expression levels in groups 1, 2, and 3 [Table 1].

Significant differences in age and sex were observed in patients who did or did not develop TMD based on their demographic data. Women were more likely than men to develop TMD. In addition, patients greater than or equal to 25 years of age were more likely to develop TMD than those less than 25 years old [Table 2].

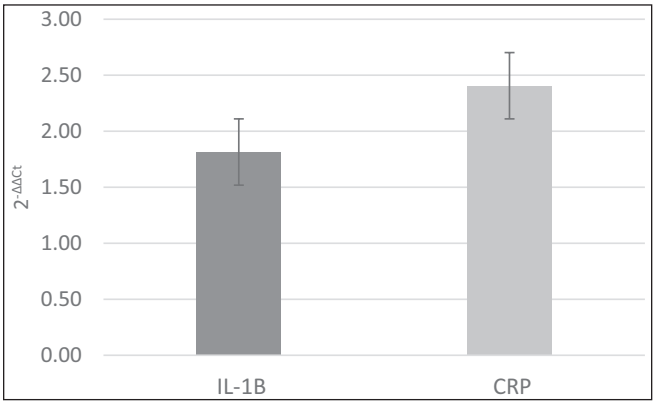

Figure 1: Relative expression of interleukin (IL)-1β and C-reactive protein (CRP) in mRNA isolated from buccal swabs of the groups with temporomandibular disorder ($n = 68$)

Discussion

The results of this study showed that women are at a higher risk of developing TMD than men at greater than 25 years of age after orthodontic treatment. Other studies have confirmed this pattern, with women being reportedly twice as likely to develop TMD than men, particularly if they are older, have undergone orthodontic treatment, or have a history of intra-articular disorders, occlusion disorders, stress, or bad habits.[27-29]

In this study, 68 participants were found to have developed TMD after orthodontic treatment using the DC/TMD classification system, an essential diagnostic tool for TMD. A similar categorization system has also been employed in other studies using the DC/TMD's Diagnostic Decision Tree; namely, muscle disorders (group 1), articular disc displacement (group 2), and cephalgia, which consists of arthralgia, osteoarthritis, or osteoarthrosis (group 3).[20,30,31]

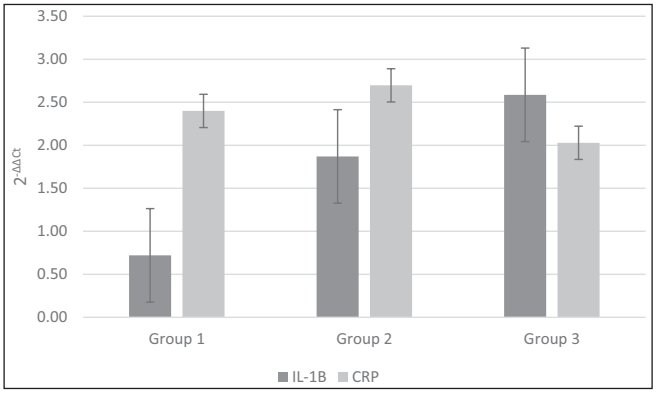

Figure 2: Relative expression of interleukin (IL)-1β and C-reactive protein (CRP) in mRNA isolated from buccal swabs of the groups with temporomandibular disorder (n = 68) in each group with TMD

Though patients may experience TMD after orthodontic treatment, the cause remains unknown. In this study, the prevalence of TMD after orthodontic treatment was 64.76%. However, this value cannot be assumed that orthodontic treatment causes TMD, as no data collection and comparison of the state of TMD at the beginning of orthodontic treatment was done. One study showed that TMD findings after orthodontic treatment with orthognathic surgery were not significantly different from those in the control group.[11] Other studies have indicated no substantial correlation between the occurrence of TMD and orthodontic treatment.[16]

This study used the RT-PCR method to evaluate the expression levels of IL-1β and CRP as biomarkers of TMD-associated inflammation in patients after orthodontic treatment. RT-PCR was chosen as the method for viewing biomarkers because it has advantages for detecting genetic material and doubling the target DNA for viewing gene expression with a high sensitivity and specificity. The measurement of biomarkers using RT-PCR is considered to be the gold standard for analyzing gene expression,[32] and other similar studies have also utilized this technique.[14,33-36]

Table 1: Value of $2^{-\Delta\Delta Ct}$ interleukin-1β and C-reactive protein in groups 1, 2, and 3

Variable	Group 1 (n =17)		Group 2 (n = 29)		Group 3 (n = 22)		P value
	Mean ± SD	Min–Max	Mean ± SD	Min–Max	Mean ± SD	Min–Max	
$2^{-\Delta\Delta Ct}$ IL-1β	0.72 ± 0.65	0.06–2.47	1.87 ± 4.37	0.01–23.11	2.59 ± 7.32	0.02–33.78	0.632
$2^{-\Delta\Delta Ct}$ CRP	2.40 ± 3.67	0.06–13.64	2.70 ± 5.10	0.03–21.92	2.03 ± 2.52	0.03–10.68	0.791

Kruskal–Wallis test, $P < 0.005$; CRP = C-reactive protein, IL-1 β = Interleukin-1β

Table 2: Relationship between demographic characteristics of non-TMD and TMD groups

Variable	Category	Group 0		Group 1		Group 2		Group 3		P value
		n	%	n	%	n	%	n	%	
Sex	Male	9	32.14	2	7.14	13	46.43	4	14.29	0.052*
	Female	28	36.36	15	19.48	16	20.78	18	23.38	
Malocclusion	Class I	24	41.38	6	10.34	16	27.59	12	20.69	0.124
	Class II	8	24.24	10	30.30	10	30.30	5	15.15	
	Class III	5	35.71	1	7.14	3	21.43	5	35.71	
Age (years)	<25	19	42.22	10	22.22	12	26.67	4	8.89	0.039*
	≥25	18	30.00	7	11.67	17	28.33	18	30.00	

*Chi-squared test, $P < 0.05$

Non-TMD (group 0), pain-related TMDs (group 1), intra-articular TMDs (group 2), and combined pain-related and intra-articular TMDs (group 3)

In this study, the highest level of *IL*-1β expression was found in group 3. Yuan *et al.*'s research indicates that the most representative inflammatory cytokine of TMD is IL-1β.[33] Other studies have also found IL-1β expression to be higher than CRP in a postorthodontic treatment group with TMD.[4,37] Similar results have also been found in IL-1β expression levels in orthodontic patients, patients with TMD, and patients with dentofacial deformity.[31] Elevated levels of IL-1β may be associated with the presence of symptoms resulting from TMJ abnormalities in patients with both pain-related and intra-articular TMDs. In contrast, low IL-1β expression was seen in the group with only pain-related TMDs. A similar pattern was observed in a study of TMD with osteoarthritis, which found higher IL-1β expression in this group compared to a normal group.[34]

IL-1β is one of the most prevalent cytokines and is the first one produced in response to the inflammation caused by excessive loading of the TMJ in TMD. The higher expression of IL-1β in group 3 may be explained by abnormalities in the synovial fluid of the TMJ articular capsule, which triggers cellular activity and is first produced in response to inflammation due to excessive loading of the TMJ. The ensuing inflammation increases pain sensitivity and simultaneously stimulates the central nervous system to release cytokines, including IL-1β.[13,38]

In this study, CRP expression was higher than IL-1β in Groups 1 and 2, those with pain-related TMDs and intra-articular TMDs, respectively. Increased CRP expression illustrates the acute phase of inflammation, as CRP is a biomarker of acute inflammation.[4,39,40] This finding is similar to studies in patients with rheumatoid arthritis of the TMJ, in whom high CRP levels were also found.[30,35] CRP levels may be closely related to TMD pain at higher levels for longer durations. Arthritis, impaired function, and bone loss in the TMJ have been shown to increase CRP expression.[39,40] The finding of high CRP expression in group 2, or in patients with intra-articular TMD, illustrates this point. However, the mechanism by which CRP biosynthesis is stimulated remains unclear.[39] This study showed that IL-1β and CRP expression levels in postorthodontic treatment patients with TMD were not significantly different between each group. However, this finding requires more in-depth research and explanation.

Conclusion

In this study, mRNA expression patterns of IL-1β and CRP were measured in patients with TMD who had undergone orthodontic treatment. It was found that patients with either pain-related or intra-articular TMDs tended to have higher CRP expression than IL-1β. However, in patients with combined pain-related and intra-articular TMDs, the IL-1β levels were higher than CRP. Women and those in adulthood were also shown to be more prone to developing TMD. This study indicates that IL-1β and CRP may have utility as biomarkers for inflammation in patients with TMD after orthodontic treatment. However, it should be noted that our findings were not statistically significant. The limitation of this study was that it solely focused on IL-1β and CRP. However, other biomarkers may be relevant for TMD, including TNF and IL-6. Another limitation is that this study did not observe previous TMD conditions and did not use subjects without orthodontic treatment.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

The author would like to thank the Director of Dental and Oral Hospital Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia, Dr Yudi Ardila Sp.BM(K) for his permission and support to use the facilities in this research. Thanks also to the laboratory assistants in the laboratory and research unit of the Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia.

Financial support and sponsorship

This research received no support or grants.

Authors contributions

NI: data collection, laboratory work, conceptualization, and manuscript preparation. EWB: conceptualization, laboratory supervision, data interpretation, and proofreading of manuscripts. MK: conceptualization, supervision, and drafting. IT: conceptualization, supervision, and drafting. EM: conceptualization, supervision, and drafting.

Ethical policy and institutional review board statement

The research protocol received approval from the Ethics Committee of The Research Faculty of Dentistry, Universitas Indonesia, under the reference number 11/Ethical Approval/FKGUI/III/2022, with protocol number 070080222.

Patient declaration of consent

The "Patient Declaration Consent" has been properly signed by all research subjects.

Data availability statement

Data can be acquired through email from the corresponding author.

List of Abbreviations

CRP: C-reactive protein
IL-1: Interleukin-1
DC/TMD: Diagnostic Criteria for Temporomandibular Disorders
TMD: Temporomandibular joint disorder
TNF: tumor necrosis factor

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