

# Salivary Interleukin-6 Levels in Patients with Periodontitis Stage IV

## *Evre IV Periodontitis Hastalarında Tükürük İnterlökin-6 Seviyeleri*

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### Abstract

**Objective:** The role of interleukin-6 (IL-6) in the pathogenesis of periodontal disease and tissue destruction at the periodontal site has been widely reported. This study aimed to evaluate the salivary IL-6 levels in patients with stage IV periodontitis.

**Materials and Methods:** The study included 28 patients who were diagnosed with periodontitis stage IV and the control group of 22 periodontally healthy patients. All the patients were systemically healthy. Saliva samples were collected, and clinical periodontal measurements, including probing depth (PD), clinical attachment level (CAL), papilla bleeding index (PBI), the percentage of sites with bleeding on probing (BOP) %, plaque index (PI) and calculus index (CI), were recorded. The unstimulated saliva of each patient was collected by a saliva collector, and all samples were analysed using the enzyme-linked immunosorbent assay method for the detection of IL-6.

**Results:** The mean value of salivary IL-6 in patients with periodontitis stage IV was  $22.18 \pm 5.96$  pg/mL. In the control group, the average measured value of IL-6 was  $2.23 \pm 2.17$  pg/mL. The periodontitis group had a significantly higher salivary IL-6 levels than the control group. A strong positive correlation was observed between the salivary IL-6 and clinical periodontal parameters (PD, CAL, PBI, BOP %, PI and CI) in patients with periodontitis stage IV ( $p < 0.0001$ ).

**Conclusion:** We demonstrated a statistically significant relationship between periodontal parameters and salivary IL-6 in patients with periodontitis stage IV. New studies are needed to accurately establish salivary IL-6 potential as a biomarker for periodontal disease monitoring, including all stages and grades of periodontitis.

### Keywords

periodontitis, saliva, interleukin-6, ELISA

### Anahtar Kelimeler

periodontit, tükürük, interlökin-6, ELISA

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### Öz

**Amaç:** İnterlökin-6'nın (IL-6) periodontal hastalık patogenezi ve periodontal doku yıkımındaki rolü geniş çapta ele alınmıştır. Çalışmanın amacı, evre IV periodontitis hastalarının tükürüklerindeki IL-6 seviyelerini değerlendirmektir.

**Gereç ve Yöntemler:** Araştırmaya evre IV periodontitis teşhisi konulan 28 hasta ile 22 hastadan oluşan kontrol grubu dahil edildi. Hastaların tümü sistemik olarak sağlıklıydı. Tükürük örnekleri toplandı ve sondalama derinliği (SD), klinik ataşman seviyesi (KAS), papil kanama indeksi (PKİ), sondalamada kanama yüzdesi (SK %), plak indeksi (Pi) ve diştaşı indeksini (Di) içeren klinik periodontal ölçümler kaydedildi. Her bir hastanın uyarılmamış tükürükleri, tükürük toplayıcı ile toplandı ve örneklerdeki IL-6 seviyesi enzim bağılı immünosorbent testi ile analiz edildi.

**Bulgular:** Evre IV periodontitis hastalarının ortalama IL-6 değeri  $22,18 \pm 5,96$  pg/mL idi. Kontrol grubunun ortalama IL-6 değeri ise  $2,23 \pm 2,17$  pg/mL idi. Periodontitis

grubunun tükürük IL-6 seviyesi kontrol grubundan daha yüksek bulundu. Evre IV periodontitis hastalarının tükürük IL-6 seviyeleri ile klinik periodontal parametreleri (SD, KAS, PKI, SK %, PI ve DI) arasında anlamlı ve güçlü pozitif korelasyonlar gözlemlendi ( $p < 0,0001$ ).  
**Sonuç:** Çalışmamız, evre IV periodontitis hastalarının tükürük IL-6 seviyeleri ile klinik periodontal parametreleri arasında istatistiksel olarak anlamlı bir ilişki olduğunu ortaya koymuştur. Periodontitisin tüm evre ve derecelerini içeren periodontal hastalık takibinde, tükürük IL-6 seviyelerinin biyobelirteç olarak tam potansiyelini tespit etmek için yeni çalışmalara ihtiyaç vardır.

## Introduction

Saliva is an biological fluid and according to recent data, it mirrors general health conditions and reflects various systemic changes in the body (1). Additionally to containing secretion of major and minor salivary glands it also contains several constituents that do not origin from salivary glands: Gingival crevicular fluid, derivatives of blood and serum, expectorated bronchial and nasal secretions, viruses, fungi, bacteria, and bacterial products, various cells, electrolytes, immunoglobulins, proteins and enzymes, food debris and a small portion is gastro-esophageal reflux, etc. (2). This makes her an important diagnostic tool.

Although bacteria are the primary cause of periodontitis, the immune-inflammatory response of the host is responsible for the most destructive changes in periodontal tissue (3). A systematic review by Kc et al. (4) recognized interleukin-6 (IL-6) together with IL-1beta (IL-1 $\beta$ ) as key biomarkers with accuracy that is acceptable for diagnostics of periodontitis. Several recent publications dealt with the potential diagnostic significance of saliva. It was assumed that saliva can be used not only for the diagnosis of oral diseases but also as a "body mirror" and can also be used to diagnose systemic illnesses (5). Saliva is an easy-to-reach body fluid that contains a wide range of significant proteins that are produced locally or transported through the blood vessels in the gingival tissue (6). Since many biomarkers in the saliva can be found in the blood, the use of saliva in the diagnosis is of great importance. The ideal biomarker must be sensitive, specific, prophetic, fast, economizing, non-invasive, and stable *in vivo* and *in vitro*. Compared to drawing blood, saliva sampling is a non-invasive and much more pleasing method for patients and is a diagnostic method suitable for children, young people, and the elderly. IL-6 is esteemed as a pro-inflammatory cytokine which acts on bone resorption in the presence of infections (7). The presence of

IL-6 can be demonstrated by saliva analysis which can be a very important diagnostic method. The measurement of some biomarkers, including IL-6, can be confusing since the source of this cytokine can be local production by circulating neutrophils due to inflammation in periodontal tissues, or due to systemic inflammation and originating from the blood and circulation.

Salivary IL-6 has been measured using enzyme-linked immunosorbent assay (ELISA), Luminex multiplex assay, and enzyme immunoassay (EIA). In the study by Ramseier et al. (8) salivary IL-6 ranged from 22.1 pg/mL in patients with gingivitis to 88.7 pg/mL in patients with moderate to severe chronic periodontitis. Ebersole et al. (9) investigated the diagnostic potential IL-6 and mean IL-6 values obtained in the saliva samples were  $3.30 \pm 2.32$  pg/mL for healthy subjects and  $35.57 \pm 48.17$  pg/mL for patients with chronic periodontitis. Mean IL-6 levels in saliva in patients with chronic periodontitis ( $98.40 \pm 18.44$  ng/L) was significantly higher than in controls ( $11.67 \pm 3.32$ ;  $p = 0.001$ ) in a study by Nanakaly (10). In the study by Nagarajan et al. (11) the mean value of salivary IL-6 in patients with gingivitis was  $3.9 \pm 5.9$  pg/mL, and  $12.1 \pm 10.2$  pg/mL in periodontitis patients who had bleeding on probing (BOP) at  $>20\%$  of sites, with  $>10\%$  of sites with probing depth (PD)  $\geq 4$  mm and clinical attachment level (CAL)  $\geq 2$  mm.

Correlation of the obtained salivary IL-6 values in patients with periodontitis stage IV and healthy subjects is intended to determine the reliability of IL-6 in saliva as an inflammatory mediator for patients with periodontitis stage IV. We would like to evaluate salivary IL-6 levels in patients with stage IV periodontitis, and compare the results to earlier findings of salivary IL-6 in periodontitis. Reliability and precision of the results should give us guidelines for the possible use of salivary IL-6 as a biomarker in everyday practice.

## Materials and Methods

### Study Groups

The study protocol was in accordance with the local ethical guidelines and following the Helsinki Declaration of Human Rights and approved by the University in Sarajevo Ethics Committee of the Faculty of Dentistry (decision no: 02-3-4-189-9, date: 22.04.2014).

The study was conducted at a private dental practice in Sarajevo, Bosnia and Herzegovina. The study included patient volunteers who agreed to participate in the study. Patients were informed of the purpose and manner of conducting the study and gave their consent to participate in the study. Patients were selected by random sampling method using a software (Research Randomizer software). Patients were classified using the 2017 classification of periodontal and peri-implant diseases and conditions (12). The test group included 28 patients who were diagnosed with generalized periodontitis stage IV. The diagnosis was performed based on the history of the disease, and clinical examination. The control group consisted of 22 patients who did not have periodontal disease. The criteria for the healthy patient was age between 18-50 years, PD  $\leq$ 3 mm, the percent of sites with papilla bleeding index (PBI)  $\geq$ 2 less than 10%, no sites with PBI  $>$ 4, and the absence of alveolar bone resorption. All patients were Caucasians, and smoking data were included in the patient's history. A sample size of 40 subjects (sample size analysis,  $d=2$ ) was calculated with a confidence interval of 95%,  $\alpha=0.05$ , and a strength of 80% using the standard deviation (SD) for the CAL parameter according to a study by Yue et al. (13).

Excluding factors were: History of periodontal therapy (non-surgical and surgical), the existence of systemic illnesses, presence of infectious diseases, need of antibiotic prophylaxis before examination, mental disability, use of antibiotic, corticoid or immunosuppressive therapy within the six months prior to the study, pregnancy, lactating women, minors, patients undergoing endodontic treatment, patients with periapical lesions, the use of antiseptics, and antimicrobial drugs.

Before taking the sample, patients gave written consent to participate in the study. History of disease and clinical examination data were entered in a

specially prepared work chart. Following parameters were recorded: plaque index [PI, Silness and Loe (14)], PBI [Saxer and Mühlemann, (15)], calculus index [CI, Silness and Loe, (14)], tooth mobility, suppuration, PD, CAL, and the engagement of the furcation [Hamp et al. (16)]. All clinical parameters were registered by the same clinician, and World Health Organization periodontal probe was used in registration.

### Sampling of Saliva

Before registration of the clinical parameters, samples of patients' saliva were collected. Saliva samples were collected for all patients on working days (Monday to Friday) between 9-10 AM.

A sample of unstimulated saliva was collected from the participants before periodontal treatment for the test group in a sterile vial of 5 mL (Thermo Scientific™, Thermo Fisher Scientific, USA) with the use of Saliva Collection Aid, by Salimetrics, USA. Saliva was collected on the volume base according to the modification of the method described by Navazesh and Kumar (17). Samples were collected from the patients in the control group without periodontal treatment required. Each participant rinsed with distilled water for one minute 10 minutes before the sampling to remove debris. This was done following the instruction of ELISA kit manufacturer Salimetrics, to minimize the possible effects of acidic or high sugar foods which can compromise assay performance by lowering sample pH and influencing bacterial growth.

After collecting, saliva samples were refrigerated within 30 minutes and frozen at  $-20$  °C within 4 hours until further analysis.

### Analysis of Saliva Samples

All samples were analyzed using the (Salimetrics 1-3602-Interleukin-6 Salivary Immunoassay Kit) ELISA/EIA manufactured by (Salimetrics), USA. We used ELISA sandwich immunoassay specially designed and validated for the quantitative measurement of salivary IL-6. Preparation of reagents, sample handling, and preparation procedures of IL-6 analysis in saliva samples were performed according to the manufacturer's instructions. The obtained results were expressed in optical density, using software and 4-parameter non-linear regression curves. The results were computed using (RayTo, Microplate Reader RT-6100). The obtained IL-6 values are expressed quantitatively in pg/mL.

### Statistical Analysis

Continuous variables were presented as mean  $\pm$  SD/median. ANOVA test and t-test were used for comparisons of means and proportions between the test group and the healthy control group. Pearson's correlation coefficient was used to assess the correlation of salivary IL-6 and clinical periodontal parameters. A two-tailed  $p < 0.05$  was considered to be statistically significant in all analyses. Empower stats software was used for data analysis.

### Results

The study included patients 18-50 years old. The mean age of patients in the test group was  $46 \pm 4.1$  and for the control group  $39 \pm 11.3$  ( $p = 0.0069$ ). The test group was formed of 14 male and 14 female patients, and the control group was formed of 22 patients, 10 male and 12 female patients. P values for male-female ratio in study groups were  $p = 0.7242$  for male patients, and  $p = 0.7230$  for female patients.

Smoking was registered and 15 patients in the test group and 14 patients in the control group were confirmed to be smokers ( $p = 0.4788$ ). Average number of extracted teeth in test group was 6.57, and the average number of extracted teeth in the

control group was 4.27 ( $p < 0.0001$ ). Average number of extracted multiple rooted teeth in the test group was 4.17, and in the control group 2.36 ( $p < 0.0001$ ).

Clinical periodontal parameters in the test group had the following values: PBI:  $3.6 \pm 0.2$ , CI:  $3.3 \pm 0.4$ , PD:  $7.1 \pm 0.6$ , BOP %:  $43.76 \pm 6.65$ , and CAL:  $5.76 \pm 1.29$ . All clinical periodontal parameters of patients in the test group had a significant difference compared to the values of the clinical periodontal parameters in the control group. Table 1 summarizes the results of demographic and clinical periodontal parameters for both groups.

The mean value of salivary IL-6 in patients with periodontitis stage IV was  $22.18 \pm 5.96$  pg/mL and the average measured value of IL-6 in the saliva of the control group was  $2.23 \pm 2.17$  pg/mL ( $p < 0.0001$ ) (Figure 1).

### Correlation Between Salivary IL-6 and Clinical Periodontal Parameters

We correlated the following clinical periodontal parameters with salivary IL-6: PI, PBI, CI, PD, BOP %, and CAL. The results show that there is a strong significant correlation between salivary IL-6 and clinical periodontal parameters in patients with periodontitis stage VI, and the results were statistically significant (Table 2).

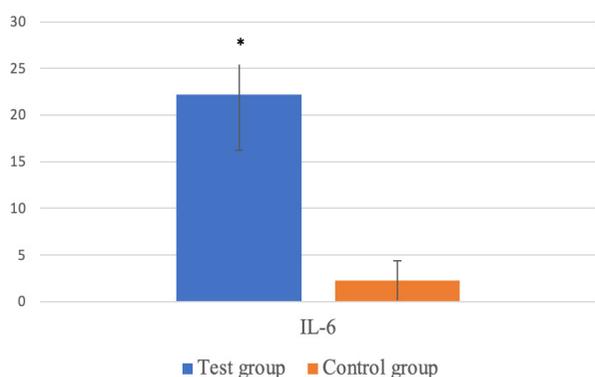
**Table 1. Demographic and clinical periodontal parameters of patients**

Parameters	Test group (n=28)	Control group (n=22)	p-value
Age, year (mean $\pm$ SD)	$46.3 \pm 4.1$	$39.8 \pm 11.3$	$p = 0.0069$
<b>Gender</b>			
Male (n)	14	10	$p = 0.7242$
Female (n)	14	12	$p = 0.7230$
PI	$2.8 \pm 0.3$	$0.3 \pm 0.2$	$p < 0.0001$
PBI	$3.6 \pm 0.2$	$0.8 \pm 0.4$	$p < 0.0001$
CI	$3.3 \pm 0.4$	$0.6 \pm 0.2$	$p < 0.0001$
PD	$7.1 \pm 0.6$	$1.6 \pm 0.6$	$p < 0.0001$
BOP (%)	$43.76 \pm 6.65$	$5.01 \pm 2.32$	$p < 0.0001$
CAL	$5.76 \pm 1.29$	$0.02 \pm 0.01$	$p < 0.0001$
<b>Smoking</b>			
Yes	15	14	$p = 0.4788$
No	13	8	
IL-6	$22.18 \pm 5.96$	$2.23 \pm 2.17$	$p < 0.0001$
PI: Plaque index (0-3), PBI: Papilla bleeding index (0-4), CI: Calculus index (0-4), PD: Pocket depth (mm), BOP (%): Bleeding on probing (%), CAL: Clinical attachment loss (mm), IL-6: Interleukin-6 in saliva (pg/mL), SD: Standard deviation			

**Table 2. Salivary IL-6 and clinical periodontal parameters correlation**

Parameter	Test group	Control group
	p-value	p-value
PI	p<0.0001	p=0.0002
PBI	p<0.0001	p=0.0041
CI	p<0.0001	p=0.0011
PD	p<0.0001	p=0,1965
BOP (%)	p<0.0001	p=0.0100
CAL	p<0.0001	p<0.0001

PI: Plaque index (0-3), PBI: Papilla bleeding index (0-4), CI: Calculus index (0-4), PD: Pocket depth (mm), BOP (%): Bleeding on probing (%), CAL: Clinical attachment loss (mm)

**Figure 1. Salivary IL-6 levels (pg/mL) in study groups\***

\*P<0.0001, bar: Mean values of salivary IL-6 in study groups, line: Standard deviation values of salivary IL-6 in study groups

IL-6:interleukin-6

The results show that there is a highly significant difference between IL-6 in saliva and clinical periodontal parameters in the test group: PD (p<0.0001), BOP % (p<0.0001), CAL (p<0.0001). In the control group, the correlation of salivary IL-6 and clinical periodontal parameters showed no significant relation to PD (p=0.1965), while the correlation was significant for BOP % (p=0.0100) and CAL (p<0.0001).

## Discussion

IL-6 has both pro-inflammatory and anti-inflammatory function and is a very important mediator of inflammation. It is, however, important to elucidate that IL-6 is a part of a complex network of cytokines included in the inflammatory response, and therefore a part of a possible complex of biomarkers. Several studies conclude that ILs are important as biomarkers to identify patients with periodontitis

(13,18,19). This study does have its downsides; we did not include patients with gingivitis so we are not able to correlate and possibly distinguish levels of IL-6 for both gingivitis and periodontitis. Also, further studies would have to include other possible biomarkers for periodontitis in order to find a sensitive and specific set of biomarkers for diagnosis and monitoring of periodontitis.

We demonstrated a statistically significant difference between IL-6 levels in saliva of patients with periodontitis stage IV and the control group. Also, the correlation of IL-6 in the saliva of patients with periodontitis stage IV and clinical parameters (PI, PBI, CI, PD, BOP, and CAL) were statistically significant. In the Ebersole et al. (20) study of 2015, four biomarkers were included, including IL-6 in patients with healthy periodontal tissues, gingivitis patients, and patients with periodontal disease diagnosis. In this study, IL-6 was the highest in the saliva of patients with periodontitis (22.8±3.7 pg/mL) compared to gingivitis (6.3±2.7 pg/mL) and patients with healthy periodontal tissue (3.7±0.5 pg/mL). In the second study, Ebersole et al. (9) investigated the diagnostic potential of IL-1β, IL-6, matrix metalloproteinase-8, and salivary albumin, and compared the values obtained in healthy subjects and patients with chronic periodontitis. The values of all four observed biomarkers, including IL-6, were significantly increased in salivary patients with chronic periodontitis and concluded that biomarkers had significant diagnostic potential for periodontal disease. The mean IL-6 values obtained in the saliva samples in this study were 3.30±2.32 pg/mL for healthy subjects and 35.57±48.17 pg/mL for patients with chronic periodontitis. In this study, Ebersole et

al. (9) have had higher IL-6 values in patients with chronic periodontitis comparing to the values we had in our study. The values obtained for healthy patients, on the other hand, are similar to those in our study. In this study, Luminex multiplex assay was used for determination of IL-6 which may have impacted the differences in the results, and also reason may reside in the level of disease of the different study populations. The periodontitis group contained significantly more men, non-Caucasians, and smokers, and was significantly older than the controls.

Nagarajan et al. (11) study measured the values of four biomarkers including IL-6 in 40 gingivitis patients and 40 patients with periodontitis. The mean value and standard deviation of IL-6 in the saliva sample for patients with periodontitis is  $12.1 \pm 10.2$  pg/mL. A study by Nanakaly (10) published in 2016 demonstrated higher salivary IL-6 levels in patients with periodontitis compared to healthy subjects. The values obtained in this study were consistent with Costa et al. (21) findings, which also demonstrated a significantly higher IL-6 value in saliva in patients with periodontitis compared to healthy patients. Significantly higher values of IL-6 in the saliva sample in patients with chronic periodontitis compared to the healthy control group were also proved by Geng et al. (22).

Higher levels of IL-6 in the saliva of patients with periodontal disease were found in a study by Teles et al. (23). In their study of 118 patients, of whom 74 were patients with chronic periodontitis, the level of IL-6 was also measured by the ELISA method. The results obtained in this study were not statistically significant because the difference in IL-6 in patients with chronic periodontitis and healthy subjects was low. A possible explanation for the discrepancy between the results might reside in the level of disease of the two different study populations. Also, in this study used cytokine levels were determined using a multiplexed bead immunoassay using Luminex, and this methodology difference used for the quantification of the IL-6 (ELISA vs. Luminex) might also have impacted the difference in the results.

The results of Ramseier et al. (8) agree with higher IL-6 in the saliva of patients with periodontitis than those in healthy subjects but the difference was small and obtained results were not statistically significant. In this study, saliva samples were supplemented with

a proteinase inhibitor combination of 1% aprotinin and 0.5% phenylmethanesulphonyl fluoride prior to storage at  $-80$  °C, which is different from our study. Also, the levels of IL-6 were measured by using protein microarray which may also be a reason for the difference in the results.

A study by Kc et al. (4) states that IL-6 is a strong salivary biomarker for periodontal inflammation with a sensitivity range of 52-80%, and specificity of 48-87%. Since periodontitis is an episodic disease with various biological stages and cyclic nature studies may have included patients with different stages which may be a reason for a different expression of IL-6 biomarker.

We demonstrated a statistically significant correlation of IL-6 values in the saliva of patients with periodontitis stage IV and periodontal parameters. Javed et al. (24) also demonstrated a statistically significant association of IL-6 levels in saliva and clinical parameters including PBI and found that IL-6 levels increased proportionally to the severity of periodontal disease. A study by Teles et al. (23) did not demonstrate a significant difference between levels of any of the 10 cytokines tested they tested and reported only weak statistically significant associations of mean clinical parameters and mean salivary levels of IL-8 and IL-10, casting a doubt on salivary cytokines as a biomarker for periodontitis. Ng et al. (25) reported a significant relation of salivary IL-6 and loss of alveolar bone, and in the study by Ebersole et al. (9), IL-6 levels were significantly positively correlated with BOP frequency in the population.

In our study, higher IL-6 values were obtained in patients with severe teeth mobility and X-ray visible resorptive changes in bone tissue. This fact is also corroborated by the evidence of Kurihara et al. (26) who have shown that IL-6 locally produces osteoclasts, which is a very important factor in the differentiation of these cells, and the role of osteoclast in bone resorption is known to us. These facts are also consistent with the findings of other studies (27,28) that speak of higher values of IL-6 as biomarkers in people with periodontal disease. The presence of plaque and dental concretions, in particular lipopolysaccharide activity on the tissue results in the activation of monocyte/T-lymphocytes. Their activation leads to increased cytokine secretion, including the IL-6 produced by multiple cells involved

in the inflammatory reaction of the organism, and periodontal disease is precisely the result of this type of reaction. These studies suggest that there are contradictions in the obtained results and measured values of IL-6 in patients with chronic periodontitis and IL-6 levels in patients compared to healthy controls. However, the IL-6 level correlation in the saliva of patients with periodontitis stage IV and the clinical parameter PBI tells us that there is a link that should be examined further. Possible directions for further investigation of IL-6 and periodontal disease bond could be in the measurement of IL-6 in the gingival crevicular fluid of patients with all grades of periodontitis using the new classification.

The limitations of this study are that the study was performed only in patients with generalized stage IV periodontitis. Patients were not divided according to the grade of the disease, which in potential studies with a larger number of participants and inclusion of all grades of the disease, could provide data on the potential impact of the disease on systemic health or the patient's response to standard therapy of periodontitis.

### Conclusion

We demonstrated a statistically significant relationship between periodontal parameters and salivary IL-6 in patients with periodontitis stage IV. This data support earlier findings of IL-6 as one of the salivary biomarkers which may be useful in monitoring the current state of the disease, the effectiveness of the treatment, and possibly predict the progression of periodontal disease. But before it could be used as a tool in clinical practice, more data on salivary IL-6 in periodontitis is needed. Since periodontitis is an episodic inflammatory disease with various biological stages and cyclic nature, studies must include patients with different stages and grades of periodontitis. We may expect different expression of IL-6 biomarker in different stages of inflammation.

### Ethics

**Ethics Committee Approval:** The study protocol was in accordance with the local ethical guidelines and following the Helsinki Declaration of Human Rights and approved by the University in Sarajevo Ethics Committee of the Faculty of Dentistry (decision no: 02-3-4-189-9, date: 22.04.2014).

**Informed Consent:** Patients were informed of the purpose and manner of conducting the study and gave their consent to participate in the study.

**Peer-review:** Externally and internally peer-reviewed.

### Authorship Contributions

Concept: Z.H., E.P., M.H., Design: Z.H., E.P., S.H., Supervision: E.P., M.H., Fundings: Z.H., M.H., Materials: M.G.V., S.H., Data Collection or Processing: M.G.V., S.H., Z.H., Analysis or Interpretation: Z.H., E.P., Literature Search: M.G.V., Writing: Z.H., Critical Review: M.G.V., S.H.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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