Original Article

# Salivary Semaphorin 4D Level in Patients with Different Severities of Periodontitis (Observational Case–Control Study)

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## Abstract

**Background:** Periodontal diseases are progressive, destructive, and inflammatory conditions of the tissues that support the teeth. An immune response is triggered by microbial dysbiosis in the sub-gingival biofilm. Semaphorin 4D (Sema4D) is a glycoprotein expressed by osteoclasts, T-cells, and activated B-cells involved in both immune response and bone remolding. **Objectives:** This study attempted to assess the salivary levels of Sema4D in patients with different severities (stages I–IV) of periodontitis in comparison to healthy control. **Materials and Methods:** A total of 171 participants of both genders were included into this case–control study. From the dental centers in Al-Najaf city and the Department of Periodontics in the College of Dentistry, University of Kufa, five groups were established for them: clinically healthy periodontium control group (19 subjects), Stage I periodontitis group (38 subjects), Stage II periodontitis group (38 subjects), Stage III periodontitis group (38 subjects), and Stage IV periodontitis group (38 subjects). The clinical periodontal parameters were examined after collecting whole unstimulated salivary samples from all individuals. The levels of Sema4D in saliva samples were estimated utilizing the ELISA technique. Statistical analysis used: ANOVA test, Games-Howell test, and Pearson correlation ($r$) test. **Results:** The findings revealed that the mean level of salivary Sema4D was the highest in the stage IV periodontitis group (758.11 ± 139.119), followed by stage III periodontitis group (725.815 ± 127.055), stage II periodontitis group (679.169 ± 121.993), stage I periodontitis group (601.742 ± 145.459), and control group (366.515 ± 47.635) respectively with a significant difference ($P \leq 0.01$). **Conclusions:** The periodontitis patients showed higher Sema4D levels in unstimulated saliva as compared to the control. Hence, this molecule may play an essential role in the pathogenesis of periodontitis.

**Keywords:** Periodontitis, saliva, semaphorin 4D

## Introduction

Periodontal diseases are progressive, destructive, and inflammatory conditions of the tissues that support the teeth.[1,2] Periodontitis is thought to be initiated by dental biofilms that accumulate below and at the gingival edge.[3] An immune response is triggered by microbial dysbiosis in the sub-gingival biofilm, and the periodontium then persists in an inflammatory state as a result of interactions between human immunity and dysbiotic microbial communities. Moreover, tissue damage results from inappropriate host immunological response. The immune and inflammatory responses of the host are critical in the pathogenesis of periodontitis.[4] Affected by genetic characteristics, environmental factors,[5] and systemic diseases such as diabetes.[6-8] The clinical periodontal parameters are the best diagnostic tools now available; however, they could only evaluate the disease's current severity and extent. No information could be obtained regarding future disease activity.[9] Saliva has long been thought to be a reflection of the overall health of the body since it includes the serum elements which are evaluated in standard blood tests for disease and health monitoring, saliva as a diagnostic fluid provides a lot of advantages over serum for disease diagnosis.[10] A salivary diagnostic test is noninvasive, sensitive, specific, and useful for evaluating large populations and

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predicting periodontal disease progression or stability.[11] Semaphorin 4D (Sema4D) is a membrane-bound protein that could be proteolytically cleaved to form soluble Sema4D (sSema4D). Activated B-cells, T-cells, mature dendritic cells (DCs), and neutrophils express Sema4D.[12] Sema4D has an essential role in immune system regulation such as B-cell activation and antibody production. Furthermore, Sema4D promotes DC activation and maturation, which in turn enhances the activation of T-cell.[13] It has been shown that Sema4D exacerbates inflammation by increasing tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production.[14] Sema4D was detected in the bone microenvironment and shown to be a product of osteoclasts that inhibits bone production by acting on osteoblasts, hence altering bone homeostasis toward resorption.[15] Furthermore, Shindo *et al*.[16] reported that activated platelet released soluble Sema4D which in turn inhibits osteoblastogenesis and enhances osteoclastogenesis. In the inflammatory bone lytic lesion of periodontitis, Sema4D may be involved in pathological bone resorption and impaired bone formation, but not in healthy bone remodeling.[17] Bastos *et al*.[18] found increased levels of Sema4D in the tissue samples obtained from advanced peri-implantitis lesions than in healthy tissue samples, due to its pro-inflammatory action, which could be detrimental to bone tissue.

To the best of our knowledge, this is the first study to assess salivary levels of Sema4D in periodontitis patients. Therefore, the purpose of this study was to compare salivary levels of Sema4D in patients with different severity of periodontitis (stages I–IV) to healthy control.

### Research question

Are the levels of salivary biomarker Sema4D as compared to healthy control associated with the severity of periodontitis?

### Hypothesis

H0: Null hypothesis: there is no significant association between salivary levels of Sema4D and severity of periodontitis in comparison to healthy controls.

H1: Alternative hypothesis: there is significant association between salivary levels of Sema4D and severity of periodontitis in comparison to healthy controls.

## Materials and Methods

### Study design

A total of 171 participants of both genders aged 30–55 years were included into this observational case–control study. Sample collection started from January 2022 to April 2022 at the Dental centers in AL-Najaf City and the Department of Periodontics in the College of Dentistry, University of Kufa.

Following a detailed explanation of the study's goals and objectives, all subjects voluntarily agreed to participate and were given informed consent forms.

An oral examination and a thorough medical and dental history were obtained for each participant. All participants were systematically healthy and had at least 20 teeth.

Patients who were not included in this study were as follows: patients who have been or are currently undergoing significant periodontal therapy, patients who have taken anti-inflammatory or antibiotic medications in the last 3 months, patients suffering from chronic systemic diseases, immunocompromised patients, smoking, women who take contraceptive pills, post-menopausal women, breastfeeding mothers and pregnant, smokers, patients with orthodontic appliances, removable dentures, implants, crowns, and bridges, and patients with soft tissue lesions like an aphthous ulcer or lichen planus.

Following the collection of saliva, a thorough periodontal examination was performed including Plaque Index (PLI) which was performed by using a disclosing agent to detect presence/absence of dental plaque, the stained surfaces were recorded as score 1 and the unstained surfaces were recorded as score 0,[19] Bleeding on Probing (BOP) was measured by inserting the periodontal probe into the bottom of the gingival crevice or pocket and gently moved along the tooth (root) surface. Those sites that bled within 30 seconds were given score (1), and those that did not bleed were given score (0).[20]

Probing pocket depth (PPD): It is defined as the distance from the gingival margin to the most apical penetration of periodontal probe inserted into the gingival crevice. Clinical attachment loss (CAL): It is defined as the distance from the cementoenamel junction to the location of the inserted probe tip. A periodontal probe (the university of Michigan O probe with Williams marking) was utilized to measure all clinical periodontal parameters. Six surfaces (four for plaque assessment) of every tooth excluding the wisdom teeth were examined. The clinical examination was done by the same examiner for all participants. The control (periodontal health) group was defined as patients who had no CAL, PPD ≤ 3 mm, BOP < 10%.[21]

According to the new classification for periodontal and peri-implant diseases and conditions, the periodontitis groups were defined as: (1)Interproximal CAL was observed at ≥2 non-adjacent teeth or (2) buccal CAL ≥ 3mm with pocketing > 3mm was observed at ≥2.[22] The observed CAL cannot be ascribed to non-periodontal causes such as: (1) dental caries extending in the cervical area of the tooth, (2) traumatic gingival recession, (3) the presence of CAL on the second molar's distal aspect and associated with malposition or extraction of a third molar, and (4) an endodontic lesion draining through the marginal periodontium.[22]

Periodontitis cases were split into stages I, II, III, and IV.[22] All periodontitis cases were generalized and unstable status (PPD ≥ 5 mm or PPD ≥ 4 mm with BOP) with no risk factors (diabetes mellitus and/or smoking). Each group consists of 38 patients, except the control group which consists of 19 patients.

### Blinding

Blinding was not applicable due to the nature of the study.

### Calibration

The periodontal parameters (PLI, BOP, PPD, and CAL) for 10 subjects were measured for both inter and intra-calibration by the researcher and skilled supervisor for inter-calibration, while for intra-calibration, the researcher measured the periodontal parameters with two hours interval between the two measurements and using intraclass correlation coefficient which is the acceptable limit is >0.75.

### Saliva collection

Before the analysis of clinical periodontal parameters, the samples of unstimulated saliva (which is often preferred because it minimizes the dilution of analytes)[23] were collected from all participants under standard conditions following the instructions cited by Tenovuo *et al.*[24] The patients must not have consumed anything except water one hour before sample collection. The patients were instructed to rinse their mouths properly to remove any debris and to wait for 1–2 minutes for the water to clear. Saliva passively drooled over the lower lip to the plain tube. The tube was labeled with the subject's number that had been previously recorded on the case sheet. The saliva was centrifuged for 20 minutes at 3000 rpm, and the resulting supernatant was aspirated into Eppendorf tubes with a micropipette and then stored at –80 °C in the laboratory's freezer until the day of analysis.

### Quantitative analysis of salivary Semaphorin 4D

The levels of Sema4D were measured in the saliva of periodontitis groups and control group by using ELISA kit Cat No. MBS2023012, My BioSource, USA according to manufacturer instructions. The absorbance was determined using a spectrophotometer plate reader (HumaReader HS, Wiesbaden, Germany).

### Sample size calculation

To calculate sample size, Sema4D was used as a primary outcome of the study. The concentration of the biomarker during health is estimated to be equal to 200.42 ± 111.70 and during periodontitis its concentration is 371.33 ± 189.10.[25] Gpower software was used to calculate sample size at 95% confidence interval and 5% error margin.

### Statistical analysis

Statistical Package for Social Science (SPSS) version 21.0 (Chicago, IL) was used to describe, analyze, and present the data. Statistical analyses are divided into two types: (1) descriptive analysis included frequency and percentages, mean, and standard deviation (SD). (2) inferential analysis included Shapiro–Wilk test, ANOVA test, Games-Howell test, and Pearson correlation (*r*) test. Level of significance: Not Significant (NS) $P > 0.05$, Significant (S) $P < 0.05$ and $P < 0.01$.

### Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 650 (in 13/9/2022) to get this approval.

## Results

Table 1 illustrates that there were statistically significant differences among groups in PLI, BOP%, PPD, and CAL ($P < 0.01$).

As presented in Table 2 the findings showed that the mean level of Sema4D increased from control to stage IV periodontitis with a significant difference ($P < 0.01$).

Following multiple pairwise comparisons among groups all results were significant difference (p<0.01) except when compared between Stage II, Stage III and Stage IV, and

**Table 1: Clinical periodontal parameters for control and periodontitis groups**

| Groups | PLI | | BOP % | | PPD | | CAL | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Control | 25.092 | 3.506 | 7.042 | 1.788 | – | – | – | – |
| Stage I | 58.920 | 7.947 | 48.669 | 5.374 | 4.145 | 0.071 | 1.488 | 0.087 |
| Stage II | 63.371 | 5.837 | 51.418 | 3.027 | 4.671 | 0.186 | 2.362 | 0.451 |
| Stage III | 65.499 | 5.449 | 53.564 | 4.514 | 5.351 | 0.281 | 4.218 | 0.208 |
| Stage IV | 67.546 | 9.104 | 54.385 | 6.124 | 5.748 | 0.162 | 6.305 | 0.237 |
| P-value | 0.000 (S) | | 0.000 (S) | | 0.000 (S) | | 0.000 (S) | |

ANOVA test was utilized

**Table 2: Salivary levels of Sema4D in study groups**

| Groups | Mean | SD | *P* value |
|---|---|---|---|
| Control | 366.515 | 47.635 | 0.000 (S) |
| Stage I | 601.742 | 145.459 | |
| Stage II | 679.169 | 121.993 | |
| Stage III | 725.815 | 127.055 | |
| Stage IV | 758.111 | 139.119 | |

ANOVA test was utilized

**Table 3: Multiple comparisons of Sema4D using Games-Howell test**

| (I) Groups | (J) Groups | *P* value |
|---|---|---|
| Control | Stage I | **0.000** (S) |
| | Stage II | **0.000** (S) |
| | Stage III | **0.000 (S)** |
| | Stage IV | **0.000** (S) |
| Stage I | Stage II | 0.098 (NS) |
| | Stage III | **0.001** (S) |
| | Stage IV | **0.000** (S) |
| Stage II | Stage III | 0.481 (NS) |
| | Stage IV | 0.075 (NS) |
| Stage III | Stage IV | 0.827 (NS) |

S, significant at $P < 0.01$

**Table 4: Correlation between periodontal parameters and salivary Sema4D by groups**

| Groups | PLI | | BOP | | PPD | | CAL | |
|---|---|---|---|---|---|---|---|---|
| | *r* | *P* | *r* | *P* | *r* | *P* | *r* | *P* |
| Control | 0.295 | 0.220 | 0.363 | 0.126 | – | – | – | – |
| Stage I | 0.066 | 0.693 | 0.186 | 0.262 | 0.111 | 0.507 | 0.123 | 0.462 |
| Stage II | 0.096 | 0.566 | 0.066 | 0.692 | 0.210 | 0.206 | 0.231 | 0.163 |
| Stage III | 0.103 | 0.537 | 0.245 | 0.138 | 0.334 | **0.040*** | 0.398 | **0.013*** |
| Stage IV | 0.167 | 0.316 | 0.022 | 0.896 | 0.404 | **0.012*** | 0.440 | **0.006**** |

Pearson correlation (*r*) test was utilized

* Significant at $P < 0.05$

** Significant at $P < 0.01$

Stage I with Stage II, these results were not significant ($P > 0.05$) [Table 3].

As presented in Table 4, in Stage III and Stage IV groups, the correlation between Sema4D and both PPD and CAL was significantly positive.

## Discussion

Since the salivary level of Sema4D was significantly higher in all periodontitis groups than in controls; therefore, the null hypothesis was rejected. Certain limitations were associated with the current study. Salivary biomarkers should be able to accurately differentiate periodontal health/disease in the whole population, regardless of their condition or the presence of risk factors. The current study did not include patients suffering from periodontitis with other risk factors such as smoking and diabetes. However, generalization of the results of this study is not advised until they are confirmed by higher evidence-based trials.

The findings of the present study have shown that the lowest mean values of PLI and BOP were found in the control healthy periodontium group and its values increased with increased severity of periodontitis with a significant difference [Table 1]. These findings were in agreement with Talib and Ahmed.[26] These results were attributable to the fact that subjects in the control group had healthy periodontium and maintained proper oral hygiene and plaque control using toothbrushes and interdental aids; in addition, microbial biofilm is regarded as an essential etiological factor in the onset of periodontal diseases.[27] As for BOP, these findings demonstrated the influence of plaque accumulation on blood circulation as well as the actual pathophysiological process that occurred more in inflamed tissues, such as increased capillary fragility and permeability, and that the severity of bleeding and the ease with which it can be provoked are dependent upon the intensity of the inflammation.[28]

Also, the findings of this study demonstrated that both PPD and CAL mean values were the highest in stage IV periodontitis group, followed by stage III periodontitis group, then stage II periodontitis group, and then stage I in which the mean values of PPD and CAL were the lowest with significant differences among the groups [Table 1]. This may be attributed to increased bacterial invasion and plaque accumulation, which result in the destruction of sulcular epithelium, junctional epithelium, and alveolar bone in periodontitis. Those findings were in agreement with Ali and Ahmed[29] who demonstrated that both PPD and CAL mean values were elevated in periodontitis with increased severity.

Regarding the salivary level of Sema4D, the findings of this study demonstrated that the level of Sema4D was significantly higher in all periodontitis groups than in controls. These findings were consistent with those of Veyisoğlu *et al*.[25] who found that the GCF total amount of Sema4D was significantly higher in patients who had gingivitis and periodontitis in comparison to the control group. They also reported that the level of Sema4D in gingival crevicular fluid was significantly reduced after non-surgical treatment. This suggests that Sema4D may be involved in the inflammatory and destruction process that occurs in periodontitis.

Some studies have discovered that Sema4D stimulates B-cell activation and antibody production. Furthermore, Sema4D promotes DC activation and maturation, which in turn enhances the activation of T-cell.[13] In addition to that, Sema4D enhances the production of TNF-α and IL-6, and it is well documented that IL-6 and TNF-α induce osteoclastogenesis via RANKL production.[30]

Furthermore, in the periodontal tissue of ligature-induced periodontitis, the production of Sema4D was shown to be elevated. Anti-Sema4DmAb, when administered systemically, inhibited bone resorption and stimulated bone formation. Anti-Sema4D mAbs primarily influenced bone remodeling in periodontitis lesions indicating that Sema4D enhances osteoclastogenesis during inflammatory bone resorption.[17]

Also, the findings of this study revealed a significantly positive correlation of Sema4D with PPD and CAL in stage III and IV periodontitis groups. These findings were consistent with those of Veyisoğlu *et al*.[25] who reported that the correlation between GCF total amount of Sema4D and periodontal parameters (BOP, PI, GI, CAL, and PPD) was significantly positive. This further supported that Sema4D may have an essential role in the pathogenicity of periodontitis.

## Conclusion

The findings of this investigation showed that periodontitis groups' salivary levels of Sema4D were significantly higher than those of a healthy control group; consequently, Sema4D may play an essential role in the pathogenesis of periodontitis.

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### Conflicts of interest

There are no conflicts of interest.

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