Comparative evaluation of Semaphorin-4D, Peptidylarginine deiminase-2 and Matrix metalloproteinase-8 levels of gingival crevicular fluid in periodontally healthy and Stage III periodontitis Smoker and non-smoker patients before and after Non-surgical periodontal therapy

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Research Article

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Abstract

Background

The aim of the study was to evaluate Semaphorin-4D (SEMA-4D), Peptidylarginine deiminase-2 (PAD-2) and Matrix metalloproteinase-8 (MMP-8) levels of Gingival crevicular uid (GCF) in periodontally healthy, stage III periodontitis smoker and non-smoker patients before and after Non-surgical periodontal therapy (NSPT).

Methods

60 patients, with an equal allotment ratio for 3 groups of periodontally healthy (Group I), Non-smokers and smokers with stage III periodontitis (Group II and Group III) were evaluated for clinical and biochemical parameters in GCF for levels of SEMA-4D, PAD-2 and MMP-8 through enzyme linked immunosorbent assay (ELISA). Patients were subjected to NSPT and the 3 months post therapy levels were examined.

Results

Group III exhibited higher values of PPD (8.06 ± 0.19 mm), CAL (8.94 ± 0.19 mm), PI (2.58 ± 0.19) while lower PBI (1.39 ± 0.19%) and GI (1.72 ± 0.19) scores as compared to Group II, which reduced significantly from baseline to 3 months in both the groups after NSPT. For SEMA-4D, PAD-2 and MMP-8 levels in GCF, minimum values were seen for Group I which increased incrementally to Group II and III. Also, the SEMA-4D, PAD-2 and MMP-8 levels in GCF significantly reduced from baseline to 3 months amongst Group II and III.

Conclusion

The results indicate increased GCF levels of SEMA-4D, PAD-2 and MMP-8 in Group II and III, thereby implying that these molecules play a crucial role in the pathogenesis of periodontal diseases.

Clinical Relevance:

SEMA-4D, PAD-2 and MMP-8 may be suitable biomarkers of the periodontal status in future.

Summary

Increased GCF levels of SEMA-4D, PAD-2 and MMP-8 and their reduction after NSPT in Group II and III, imply that the molecules play a crucial role in the pathogenesis of periodontal diseases.

Introduction

Periodontal diseases are a consortium of inflammatory conditions affecting the supporting structures of the teeth and are primarily initiated by virulent microorganisms present within the dental plaque[1, 2].
Destruction induced in periodontal diseases is derived directly through combination mechanisms involving the microbial toxins and the activated immune system. In most of the instances the severity of periodontal destruction corresponds with the quantity and quality of microbial plaque[3, 4]. In addition, periodontal diseases are known to be influenced by multiple factors of local, environmental and genetic origins which are capable of affecting the typical slow and moderate rate of progression. Additionally, smoking is one of the commonest and strongest modifiable risk factor subsequent to dental plaque. Smokers harbor higher amounts of periodontal pathogens consequently leading to an increased severity of the disease manifested as greater clinical attachment loss associated with bone destruction as compared to non smokers. Depending on these factors the disease precipitates in to active and quiescent inflammatory periods which are important in diagnosing the cases.

Currently the diagnosis of periodontitis is based on the clinical parameters of probing pocket depth (PPD), clinical attachment levels (CAL), bleeding on probing (BOP) and the associated radiographic features. However, these methods of diagnosis are mired with inconsistencies as the parameters are a reflection of the destruction which has occurred in the past and do not necessarily exhibit the present condition nor do they predict the future progression[5, 6]. It is desirable to have diagnostic method which not only diagnoses the present condition but also provides vital information on the clinical course of the disease in future. Focusing on this research requirement many investigators have put in efforts to identify biomarkers which will provide the needed information and enable an early diagnosis consequently followed by adequate intervention.

Recent preliminary investigations have suggested that Semaphorins and peptidylarginine deiminases (PAD's) are related to periodontal diseases and could be used as potential diagnostic markers[7]. Several glycoproteins and their components are known to have functions which may promote the inflammatory process within the tissues. Semaphorins are one such family of cell surface glycoproteins secreted by cells of the immune system and having critical functions of angiogenesis, bone metabolism, tumor metastasis and regulation of the host immune responses[8, 9]. These molecules are also shown to be responsible for the cell to cell communications between osteoblasts and osteoclasts. Semaphorin 4D (SEMA-4D) was one of the first such glycoprotein produced by the T-cells and shown to be expressed on immune cells[10]. It is estimated that SEMA-4D stimulates the production of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) which further aggravates inflammation. On the other hand PAD’s family of enzymes initiates citrullination process which has an active role in the auto immune diseases and inflammation[11–13]. In response to the citrullinated proteins the host produces anti citrullinated protein antibodies and periodontal diseases are thought to act as a mechanism for such production. Based on this concept a positive correlation has been exhibited with citrullinated proteins formed in periodontal diseases being similar to the rheumatoid arthritis patients[14, 15]. Amongst the 5 members of the PAD family PAD-2 has been reported to be increased in periodontitis patients as compared to the periodontally healthy group[16].

One of the main types of collagenase in gingival crevicular fluid (GCF) of periodontitis patients which is known to be responsible for major collagenolytic activity is Matrix metalloproteinases-8 (MMP-8). Accordingly the MMP-8 activity in periodontitis is higher than the normal periodontium[17, 18]. These trials have provided the basis for furthering our knowledge with regards to these substances being utilized as
biomarkers in periodontal diagnosis and their correlation with each other. There is a distinct scarcity of literature available examining the association between SEMA-4D, PAD-2 and MMP-8 in periodontally healthy and stage III periodontitis smoker and non-smoker patients. Also, if the preliminary studies indicate towards increase of these substance levels in diseased state, it will be interesting to note whether their levels are restored or reduced to some extent post non-surgical periodontal therapy (NSPT) in such patients. So this clinical and biochemical trial was planned to evaluate SEMA-4D, PAD-2 and MMP-8 levels of gingival crevicular fluid in periodontally healthy and stage III periodontitis smoker and non-smoker patients before and after NSPT.

**Materials And Methods**

This interventional study was conducted from September 2020 to December 2021, and the patients were recruited from the outpatient Department of Periodontology, VSPM Dental College and Research Centre, Nagpur, India in accordance with the Helsinki Declaration of 1975 as revised in 2013. Clinical Trial Registry—India (CTRI), the primary register of the WHO International Clinical Trials Registry Platform, was used to register this clinical trial. Registration number CTRI/2020/03/024346 was assigned for this trial. After objectives and the procedure of the trial were explained, a written informed consent was obtained from the patients willing to participate. The study was also approved by Institutional Ethics Committee of our institute.

Referring to the study by Veyisoglu G. et al. (2019)[7], the authors reported mean levels of SEMA-4D, PAD-2 and MMP-8 in GCF in control, gingivitis and chronic periodontitis (CP) patients. These data were used to estimate the effect size. Based on the means for SEMA-4D across three patient groups, the effect size was 0.4843, while for PAD-2 was 0.3735 and for MMP 8 was 0.5539. The proposed study also has three groups namely: Periodontally healthy, stage III periodontitis non-smokers and stage III periodontitis smokers. An effect size of 0.45 was considered appropriate, which resulted into a sample size of 51 (17 per group) patients to provide the desired effect with 80% power and 95% confidence level. Hence, 60 patients aged between 25 to 60 years was the finalized sample size for this trial with all the participants having ≥ 20 teeth present in the oral cavity.

The periodontal diagnosis was based on the new classification for periodontal and peri-implant diseases and conditions given in 2017[19, 20]. Thus 20 patients in each group were allotted with Group 1 comprising of periodontally healthy patients, Group 2 and Group 3 comprising of untreated Stage III periodontitis non-smoker and smoker patients respectively (Fig. 1). The allotted patients had to fulfill the following criteria based on the group of allotment as

**Inclusion Criteria:**

**Group I**

Periodontally healthy patients with no signs of periodontal disease and no history of smoking.
**Group II**

Non-smoker patients with stage III periodontitis as assessed by clinical findings of PPD $\geq 5$ mm and CAL $\geq 4$ mm. ($\geq 30\%$ of teeth affected) and with radiographic evidence of bone loss and with no history of smoking.

**Group III**

Smoker patients with stage III periodontitis who were current smokers with untreated stage III periodontitis, as assessed by clinical findings of PPD $\geq 5$ mm and CAL $\geq 4$ mm. ($\geq 30\%$ of teeth affected) and with radiographic evidence of bone loss and with history of smoking at least 10 cigarettes per day for the last 3 years.

**Exclusion Criteria:**

Patients with any systemic disease, pregnant and/or lactating females, use of antibiotics within the last 6 months and anti-inflammatory drugs within the last 3 months, use of steroids or any immunosuppressive therapy, those who had symptoms of acute illness or had undergone any type of periodontal therapy or oral prophylaxis in past 6 months were excluded from the study.

**Clinical Examination:**

For systematic recording of the findings in each of the study participant, case history was recorded and the clinical and radiographic examinations were carried out. The smokers’ status was determined by verbal questioning and smokers were enrolled if they regularly smoked 10 cigarettes per day while the non-smokers were selected only if they did not smoke cigarettes in their entire lifetime. A single examiner (Al) used a manual periodontal probe to do an intraoral examination on 10 patients prior to the study. PPD, CAL, Plaque Index (PI)[21], Gingival Index (GI)[22] and Papillary Bleeding Index (PBI)[23] score were the clinical parameters examined. The measurements were taken at six different locations around each tooth and then rounded up to the nearest 0.5 mm. Readings were repeated by the same examiner (AI) two hours after the first measurement in order to perform intra observer reproducibility analysis. Intra-class correlation (ICC) coefficient with a two-way mixed effects model was obtained for each periodontal parameter. The ICC ranged from 0.92 to 0.99 in the groups ($P < 0.0001$), indicating excellent intra observer reliability.

**Gingival Crevicular Fluid Collection:**

The selected site was air dried and isolated with sterile cotton rolls. The supragingival plaque was removed gently with a sterile curette without touching the gingival margin. The site showing the greatest signs of inflammation and CAL was selected for sampling. The microcapillary pipette was placed at the entrance of the gingival sulcus, and GCF was collected. Sites that did not express any GCF or were contaminated with blood and saliva were excluded from the study. Approximately 10 µL of GCF was collected from the patients.
with stage III periodontitis and 4–5 µL from patients with healthy periodontium[24]. Collected GCF samples were immediately transferred to airtight plastic vials (Eppendorf tubes) and stored at −20°C until final assay. GCF was assayed for the laboratory markers SEMA-4D, PAD-2 and MMP-8 at baseline and at three months using commercially available enzyme-linked immunosorbent assay (ELISA Kit)§. Samples were analyzed according to the instruction manual at the Department of Biochemistry, NKP Salve Institute of Medical Sciences, Nagpur, India. Briefly GCF samples were diluted with dilution buffer in the kit and the amount of SEMA-4D, PAD-2, MMP-8 were determined. All samples have been run in duplication.

Non-surgical Periodontal Therapy (Nspt)

All patients underwent NSPT, which included scaling and root planing (SRP) performed by a single operator (RK), along with oral hygiene instructions. On recall visits every month up to 3 months, supragingival plaque was removed, and oral hygiene instructions were reinforced and clinical parameters recorded.

Statistical analysis:

The descriptive statistics for the levels of periodontal parameters were obtained in terms of mean and standard deviation across study groups. The comparison of means was carried out using one-way analysis of variance (ANOVA). Paired t-test was applied to assess the difference from baseline to 3 months, unpaired t-test was applied to assess the difference between two groups. One-way ANOVA test assessed the difference between three groups at baseline and 3 months which was followed by post-hoc Tukey test. All the data analyses were performed using SPSS software and the statistical significance will be tested at 5% level.

Footnotes

† PCPUNC 15; Hu Friedy, Chicago, IL, USA.

‡ Labo Glass Scientific Supply, Haryana, India.

§ Kinesis Dx ELISA Kits, Los Angeles, USA

SPSS ver. 20.0 (IBM corp. USA)

Results

The patient characteristics for the periodontal and biochemical parameters of patients in all the three study groups giving the mean and standard deviation at baseline are depicted in Table 1. All the periodontal and biochemical parameters differed significantly across the three study groups with p-values < 0.0001.
Table 1
Patient characteristics, periodontal and biochemical parameters of study groups at baseline.
(mean ± SD)

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>33.5 ± 6.04</td>
<td>43.97 ± 5.68</td>
<td>49.06 ± 7.33</td>
<td>0.00</td>
</tr>
<tr>
<td>Sex (Males/females)</td>
<td>9/11</td>
<td>12/8</td>
<td>20/0</td>
<td></td>
</tr>
<tr>
<td><strong>Periodontal Parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI</td>
<td>0.41 ± 0.19</td>
<td>2.15 ± 0.19</td>
<td>1.72 ± 0.19</td>
<td>0.0001*</td>
</tr>
<tr>
<td>PI</td>
<td>0.61 ± 0.30</td>
<td>1.83 ± 0.19</td>
<td>2.58 ± 0.19</td>
<td>0.0001*</td>
</tr>
<tr>
<td>PPD (mm)</td>
<td>1.33 ± 0.31</td>
<td>6.86 ± 0.19</td>
<td>8.06 ± 0.19</td>
<td>0.0001*</td>
</tr>
<tr>
<td>CAL (mm)</td>
<td>0.00 ± 0.00</td>
<td>7.07 ± 0.19</td>
<td>8.94 ± 0.19</td>
<td>0.0001*</td>
</tr>
<tr>
<td>PBI (%)</td>
<td>0.03 ± 0.03</td>
<td>2.25 ± 0.19</td>
<td>1.39 ± 0.19</td>
<td>0.0001*</td>
</tr>
<tr>
<td><strong>Biochemical Parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEMA 4D (ng/mL)</td>
<td>200.91 ± 1.49</td>
<td>373.11 ± 1.42</td>
<td>465.76 ± 1.42</td>
<td>0.0001*</td>
</tr>
<tr>
<td>PAD 2 (pg/mL)</td>
<td>4300.30 ± 30</td>
<td>5225.95 ± 111.01</td>
<td>5525.34 ± 111.00</td>
<td>0.0001*</td>
</tr>
<tr>
<td>MMP 8 (ng/mL)</td>
<td>11.93 ± 0.75</td>
<td>35.90 ± 0.89</td>
<td>40.67 ± 0.90</td>
<td>0.0001*</td>
</tr>
</tbody>
</table>

*Statistically significant at p < 0.05.

GI - Gingival index
PI – Plaque Index
PPD - Probing pocket depth in millimeters
CAL - Clinical attachment level in millimeters
PBI - Papillary bleeding Index in percentage
SEMA 4D - Semaphorin 4D in nanograms per milliliter
PAD 2 - Peptidylarginine deiminase-2 in picograms per milliliter
MMP 8 - Matrix metalloproteinase-8 in nanograms per milliliter

The baseline and 3 months values, along with the mean difference for Group II and Group III are depicted in Table 2. It is evident that Group III patients exhibited higher values of PPD (8.06 ± 0.19 mm), CAL (8.94 ± 0.19 mm), PI (2.58 ± 0.19) while lower PBI (1.39 ± 0.19%) and GI (1.72 ± 0.19) scores as compared to Group II patients wherein these parameters were PPD (6.86 ± 0.19 mm), CAL (7.07 ± 0.19 mm), PI (1.83 ± 0.19), PBI (2.25 ± 0.19%) and GI (2.15 ± 0.19). The scores for all these parameters reduced significantly from baseline to three months in both the groups. The PPD and CAL values reduced to 4.10 ± 0.19 mm and 4.88 ± 0.23
mm in Group II while these values were 5.60 ± 0.19 mm and 6.85 ± 0.19 mm in Group III accounting to a mean reduction of 2.76 mm and 2.20 mm in Group II and 2.46 mm and 2.09 mm in Group III respectively.

### Table 2
Clinical and biochemical parameters before and after phase 1 periodontal therapy in group II and III (mean ± SD)

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 3 Months</td>
<td>Mean diff p Value</td>
</tr>
<tr>
<td>GI</td>
<td>2.15 ± 0.19</td>
<td>1.10 ± 0.19</td>
</tr>
<tr>
<td>PI</td>
<td>1.83 ± 0.19</td>
<td>0.54 ± 0.18</td>
</tr>
<tr>
<td>PPD (mm)</td>
<td>6.86 ± 0.19</td>
<td>4.10 ± 0.19</td>
</tr>
<tr>
<td>CAL (mm)</td>
<td>7.07 ± 0.19</td>
<td>4.88 ± 0.23</td>
</tr>
<tr>
<td>PBI (%)</td>
<td>2.25 ± 0.19</td>
<td>0.28 ± 0.15</td>
</tr>
</tbody>
</table>

**Biochemical parameters**

<table>
<thead>
<tr>
<th></th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEMA 4D (ng/mL)</td>
<td>373.11 ± 1.42</td>
<td>272.38 ± 1.49</td>
</tr>
<tr>
<td>PAD 2 (pg/mL)</td>
<td>5225.95 ± 111.01</td>
<td>5054.58 ± 111.01</td>
</tr>
<tr>
<td>MMP 8 (ng/mL)</td>
<td>35.90 ± 0.89</td>
<td>26.58 ± 0.75</td>
</tr>
</tbody>
</table>

*Statistically significant at p < 0.05.

GI - Gingival index

PI – Plaque Index

PPD - Probing pocket depth in millimeters

CAL - Clinical attachment level in millimeters

PBI - Papillary bleeding Index in percentage

SEMA 4D - Semaphorin 4D in nanograms per milliliter

PAD 2 - Peptidylarginine deiminase-2 in picograms per milliliter

MMP 8 - Matrix metalloproteinase-8 in nanograms per milliliter
The mean SEMA-4D, PAD-2 and MMP-8 values at baseline for Group II were 373.11 ± 1.42 ng/mL, 5225.95 ± 111.01 pg/mL and 35.90 ± .89 ng/mL while at 3 months post therapy these values reduced to 272.38 ± 1.49 ng/mL, 5054.58 ± 111.01 pg/mL and 26.58 ± 0.75 ng/mL respectively. For Group III, these values were 465.76 ± 1.42, 5525.34 ± 111.00 and 40.67 ± 0.90 which reduced post therapy to 279.99 ± 1.49, 5138.16 ± 111.01 and 31.35 ± 0.75 respectively. The differences in values of all the biochemical parameters yielded significant results with p-values < 0.0001.

Overall, with respect to the parameters like GI, PI, PPD, CAL and PBI; the scores significantly reduced from baseline to 3 months in both Group II and Group III. The difference between the parameters at baseline was significant with higher scores among Group III followed by Group II and the least among Group I except for GI and PBI where these values for Group II were greater than Group III. Similarly, at 3 months, the difference in the PI, PPD, CAL was significantly higher between the groups with higher scores among Group III while lower PBI, and GI scores as compared to Group II and the least among Group I.

Table 3 exhibits the mean differences in SEMA-4D, PAD-2 and MMP-8 between the groups at baseline and three months post therapy using post hoc Tukey test. All the parameters differed across the groups with minimum values seen for Group I which increased incrementally to Group II and III. Also, the SEMA-4D, PAD-2 and MMP-8 significantly reduced from baseline to 3 months amongst Group II and Group III. At baseline the difference among the three groups for SEMA-4D was 172.19 ng/mL, 264.83 ng/mL and 92.64 ng/mL for PAD-2 it was 925.65 pg/mL, 1225.03 pg/mL and 299.38 pg/mL while for MMP-8 it was 23.97 ng/mL, 28.74 ng/mL and 4.77 ng/mL respectively. Similarly at three months, the difference values for SEMA-4D were 71.46 ng/mL, 79.07 ng/mL and 7.61 ng/mL for PAD-2 it was 754.28 pg/mL, 837.86 pg/mL and 83.58 pg/mL while for MMP-8 it was 14.65 ng/mL, 19.42 ng/mL and 4.77 ng/mL respectively. Major difference was found between Group I and Group III followed by Group II.
Table 3
Post hoc test for difference in parameters between the groups at baseline and 3 months

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Baseline</th>
<th>3 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean Difference (p)</td>
<td>Mean Difference (p)</td>
</tr>
<tr>
<td>SEMA 4D (ng/mL)</td>
<td>Group I</td>
<td>Group II</td>
<td>172.19</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>Group I</td>
<td>264.83</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>Group I</td>
<td>172.19</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>Group I</td>
<td>92.64</td>
</tr>
<tr>
<td>PAD 2 (pg/mL)</td>
<td>Group I</td>
<td>Group II</td>
<td>925.65</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>Group I</td>
<td>1225.03</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>Group I</td>
<td>925.65</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>Group I</td>
<td>299.38</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>Group I</td>
<td>1225.03</td>
</tr>
<tr>
<td>MMP8 (ng/mL)</td>
<td>Group I</td>
<td>Group II</td>
<td>23.97</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>Group I</td>
<td>28.74</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>Group I</td>
<td>23.97</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>Group I</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>Group I</td>
<td>28.74</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>Group I</td>
<td>4.77</td>
</tr>
</tbody>
</table>

*Significance at p < 0.05

SEMA 4D - Semaphorin 4D in nanograms per milliliter

PAD 2 - Peptidylarginine deiminase-2 in picograms per milliliter

MMP 8 - Matrix metalloproteinase-8 in nanograms per milliliter
On comparison of the baseline to three months values in Group II the difference for SEMA-4D, PAD-2 and MMP-8 values were 100.73 ng/mL, 171.36 pg/mL and 9.32 ng/mL. Similarly for Group III the difference in values for SEMA-4D, PAD-2 and MMP-8 values were 185.86 ng/mL, 387.17 pg/mL and 9.32 ng/mL. the difference in values were more pronounced for Group III as compared to Group II.

Discussion

The present study evaluated SEMA-4D, PAD-2 and MMP-8 levels of GCF in periodontally healthy and stage III periodontitis smoker and non-smoker patients before and after NSPT. A few initial reports have investigated the role of these agents and have reported the increased levels of SEMA-4D, PAD-2 and MMP-8 which was correlated to the inflammatory process within the periodontal tissues[7]. It has been accepted in the literature that periodontitis can provoke systemic inflammatory response through the ulcerated pocket epithelium and vice versa wherein systemic inflammation can act as a provocation for the periodontal destruction[25, 26]. On the other hand, smoking is an important modifiable environmental risk factor for periodontal diseases which results in worsening the destructive process leading to severe forms of periodontitis[27]. Considering the above background, it was reasoned to investigate whether smoking had any influence over the SEMA-4D, PAD-2 and MMP-8 levels in periodontitis patients and to examine the response of NSPT on the levels on these parameters.

Spencer et al.[28] demonstrated that a subset of semaphorins were dynamically regulated in the periodontal ligament and alterations in semaphorins may play a fundamental role in periodontal remodelling, affecting angiogenesis or periodontal ligament cell invasion into injury sites, when considering the relationship between semaphorins and periodontium. SEMA-4D is a semaphorin that plays an important function in the immune system which has been demonstrated to be increased in inflammation and in turn stimulates TNF-α and IL-6 production further worsening the condition[10]. In one of the clinical trials, Bastos et al.[29] reported that samples taken from progressive peri-implantitis patients with bone destruction and BOP, the SEMA-4D levels were increased than the patients with healthy implants. The results of the present clinical study are in accordance to the above findings where the GCF total levels of SEMA-4D along with PAD-2 and MMP-8 were found to be elevated in the Group II and Group III indicating greater amount of inflammatory load and destruction present. The SEMA-4D levels obtained in the previous study by Veyisoglu G et al.[7] corresponds with that obtained by us, however an additional feature of this study indicates that in stage III periodontitis smoker patients the levels were further elevated. One of the contrasting features in both the studies is that the former study has reported SEMA-4D levels to be higher in gingivitis as compared to the chronic periodontitis group, though in both the groups the levels were greater than control group. The authors have not provided any explanation to this aspect, but it could be due to the time of sampling of GCF which may have been procured in the stage of quiescence. In the said study, the authors have divided groups as active and passive taking in to account the BOP present in the case. Though we did not distinguish the participants in our study based on BOP, however the strict inclusion criteria adopted probably removed the chances of inactive lesions and the SEMA-4D levels obtained indicated an increasing severity of inflammation and destruction within the tissues from Group I to Group III. The post therapy GCF levels of SEMA-4D were reduced considerably both in Group II and III but they did not match to the levels of Group I.
as stage III periodontitis patients do respond to NSPT initially to a certain extent, however the complete resolution does not take place until a definitive surgical procedure is performed.

PAD-2 expression was also found to be linked to the severity of inflammation in rheumatoid arthritis patient’s synovial fluid and was considered to be related with disease severity, circulating C-reactive protein (CRP) & anti citrullinated protein antibody levels[13]. It has been revealed that periodontitis stroma exhibits a higher presence of citrullinated protein being an inflammation-dependent process. There was an increased expression of PAD-2 levels observed in gingival connective tissue of patients with periodontitis compared with the periodontally healthy group, according to Engstrom et al.[16] Given the similarities in the pathophysiology of rheumatoid arthritis and periodontitis, a near certainty to this phenomenon was contemplated and the findings obtained in the present study are suggestive of the same phenomenon. The GCF levels of PAD-2 followed a similar trend to the levels of SEMA-4D with the maximum being associated with Group III followed by Group II and the least in Group I. These results are in agreement with the previous trials by VeyISOĞLU G et al.7 and Engstrom et al.[16] The justification for such an increase in the levels of PAD-2 in GCF must be due to tobacco smoking which causes citrullination of tissue proteins, which is considered to be the fundamental mechanism by which smoking causes and worsens auto-immune diseases[30]. Another mechanism through which this hazardous effect of smoking occurs was postulated by Kobayashi et al.[31] wherein the authors found that tobacco smoking triggers an inflammatory cytokine response, particularly IL-1β and TNF-α and higher PAD-2 expression and activity which reinforces the findings of our study and could endorse the concept that immunity has a role in periodontal disease. The reduction of PAD-2 levels post NSPT and their positive correlation with the clinical parameters additionally substantiate the observation and role of this enzyme in the inflammatory process based on which intervention strategies can be devised.

Following a similar trend the GCF levels of MMP-8 were seen to be elevated in Group II and III with the maximum levels in Group III. Though the clinical inflammatory manifestations in smokers are a bit subdued as compared to the non smokers, however there is no doubt about the presence of inflammatory process within the tissues which has lead to the breakdown except for them to be expressed in terms of redness of the tissues and BOP. In fact the destruction observed in such patients is on a much larger scale and depicts the worsening condition. The probable explanation for such a behavior is that the nicotine in cigarette smoke affects the host inflammatory response to oral pathogens thereby up regulating the release of prostaglandins and cytokines which causes the severe destruction[6]. The elevated levels of MMP-8 and their reduction post therapy are in concurrence with the previous trials[32, 33].

The present study thus confirms a positive association of SEMA-4D, PAD-2 and MMP-8 levels in GCF with the clinical status indicating severity of inflammation and periodontal tissue destruction in non smokers and smoker stage III periodontitis patients. The reduction in values of these parameters post NSPT is an indication towards decline of the levels with resolution of inflammation to a certain extent. However, there are some limitations to the present study such as study participants were not aged matched especially in Group I and the other groups, active and passive lesions were not differentiated in Group II and III which could have led to minimal inconsistency in values.
Conclusion

In conclusion and within limitations the results of the present study suggest increased GCF levels of SEMA-4D, PAD-2 and MMP-8 and their reduction after NSPT in Group II and III patients thereby indicating that these molecules play a crucial role in the pathogenesis of periodontal diseases. The greater values of these molecules in Group III as compared to Group II imply that smoking exacerbates the disease manifestations leading to worsening of the condition. The clinical features associated with all the three study groups correlate with the biochemical findings thus confirming the positive association. With further substantiation and better understanding these molecules can be considered as suitable contenders to be labeled as biomarkers of the periodontal status in future.

Declarations

Ethical Approval

All procedures performed in the studies involving human participants complied with the ethical standards of the Institutional Ethics Committee of our institute and with the Helsinki Declaration of 1975 as revised in 2013.

Authors' contributions

Dr. Aishwarya Ikhar and Dr. Rajashri Kolte contributed to Conception and design, investigations, or data acquisition, analysis, or interpretation of data, writing original draft.

Dr. Abhay Kolte and Dr. Neha Deshpande contributed to Investigations, methodology, supervision, drafting of the work and revising it critically for important intellectual content and final approval of version to be published.

Dr. Rahul Dahake contributed to editing and critically revising the manuscript.

All authors gave final approval and agreed to be accountable for the work done, ensuring its integrity and accuracy.

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References


**Figures**

**Image not available with this version**

**Figure 1**

Study Flow Chart