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Soluble Neuropilin-1 in gingival crevicular fluid from periodontitis patients: An exploratory cross-sectional study

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ABSTRACT

Background: Soluble Neuropilin-1 (sNRP-1) is a glycoprotein with angiogenic and immune regulatory functions, which correspond to processes deeply involved with periodontal diseases. This study's objective was to determine the concentration of sNRP-1 in gingival crevicular fluid (GCF) samples of severe periodontitis (stages III-IV) compared to mild-moderate (stages I-II) periodontitis patients.
Materials and methods: An exploratory cross-sectional study was conducted, including 36 adults subjected to a complete periodontal exam, which recorded the following periodontal parameters: periodontal probing depth (PPD), clinical attachment loss (CAL), bleeding on probing (BOP), gingival index (GI) and periodontal inflamed surface area (PISA). Periodontitis was defined by two periodontists using the case definition proposed by the 2017 World Workshop for periodontal diseases. GCF samples were collected to determine the levels of sNRP-1 via ELISA. The results were analyzed using descriptive statistics, Mann-Whitney, Kruskal Wallis, and Spearman tests.
Results: The levels of sNRP-1 in patient's GCF with periodontitis in stages III-IV showed a median of 38.385 ng/mL (iqr 30.98), in comparison with 20.085 ng/mL (iqr 12.74) for stages I-II ($p = 0.202$). Regardless of the periodontitis stage, we observed a positive correlation between the levels of sNRP-1 in BOP (Rho: 0.45; $p = 0.0048$), PISA (Rho: 0.50; $p = 0.0019$), PD (Rho: 0.3; $p = 0.015$) and GI (Rho: 0.37; $p = 0.02$).
Conclusions: The GCF-sNRP-1 concentration was positively related to periodontal clinical inflammatory parameters and probably could be involved in pro-inflammatory and angiogenic mechanisms observed in periodontitis. Additional studies are necessary to confirm these preliminary results.

1. Introduction

The American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) have recently classified periodontal diseases and conditions into three main groups: periodontal health, gingival diseases and disorders; periodontitis; and other conditions affecting the periodontium.[1] Periodontitis is a microbially-associated, host-mediated inflammation resulting in loss of periodontal attachment and teeth. The clinical attachment loss (CAL) is diagnosed by the circumferential assessment of erupted teeth using a standardized periodontal probe regarding the cement-enamel junction (CEJ). Periodontal diseases are common, and their prevalence varies in different populations.[2] An epidemiological study in Chile reported periodontitis in 93.45% of adults aged between 35 and 44 years of age and 97.58% of adults between 65 and 77 years of age.[3]

For several years, diagnosing periodontitis has been based on radiological and clinical findings. However, this could be improved by molecular techniques, which allows the analysis of the individual's inflammatory response and facilitates identifying different mediators, cells, or genetic material synthesized and released during the periodontal inflammation's distinct mechanisms. This new approach could result in a more accurate diagnose of periodontal pockets with progressive tissue destruction.[4] Besides the chronic inflammation mechanisms, angiogenesis could also have a relevant role during periodontal tissue destruction and periodontitis progression.[5]

Neuropilins (NRPs) are transmembrane glycoproteins playing angiogenic and immune regulatory functions.[6,7] Specifically, Neuropilin-1 acts as a receptor for the Vascular Endothelial Growth Factor-A (VEGF-A) and for Semaphorin 3A (SEMA3A), which actively participates in angiogenic, arteriogenesis, and vascular permeability

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processes.[8] Also, NRPs regulate many other functions involved in development, migration, recruitment, intercellular communication, and immunity regulation during homeostasis and disease.[9] NRP-1 mediates antigen presentation, strengthening the interaction between dendritic cells (DCs) and T cells.[7]

The last workshop for classifying periodontal diseases proposes that inflammatory biomarkers should be researched for an earlier and more accurate diagnose of periodontitis. Among them, using different inflammatory involved molecules has suggested. Thus, the new classification model proposes investigating and validating new biomarkers with clinical applicability for periodontitis monitoring.[10]

Consequently, our research question was if there are differences among the GCF concentrations of sNRP-1 in periodontitis patients according to the disease's severity. Our main objective was to determine the presence of sNRP-1, quantify sNRP-1 in GCF in periodontitis patients, and associate them with the disease's stage/severity.

2. Material and methods

2.1. Subjects

An exploratory cross-sectional study was performed with 36 individuals attending the Department of Periodontology of the Faculty of Dentistry at the Universidad de Los Andes in Santiago, Chile. All subjects signed the informed consent document approved by the Ethical Committee of the same University. Adults of both genders, presenting at least 12 teeth (excluding third molars), were included in the study. Patients were excluded from the research if they use antibiotic treatment, oral mouthwashes, or receive periodontal therapy, or instructed oral hygiene protocol (including tongue cleaning) during the last six months.

2.2. Periodontal evaluation and other variables

Two periodontists were trained to obtain an intra and inter-examiner calibration (intraclass correlation over 0.88). All patients were subjected to panoramic radiography, including bitewing, depending on the case. Then we register sociodemographic variables, medical history, and a full mouth periodontal exam (using North Carolina probe HuFriedy®, USA), evaluating six sites per tooth, and recording the following parameters: Probing depth (PD), Clinical attachment loss (CAL), Bleeding on probing (BOP), Periodontal inflamed surface area (PISA)[11] and Gingival index (GI).

To determine the stage and degree of the disease, we used the classification of periodontal diseases and conditions of 2017, defining a patient with periodontitis who presents detectable CAL in at least two teeth or a CAL equal or over 3 mm with an associated tooth pocket bigger than 3 mm.[10]

2.3. Collection of GCF

GCF samples were collected under a relative isolating condition. The supragingival plaque was carefully removed and dried using a triple air syringe. Then, four sterile absorbent paper strips were inserted in each quadrant's deepest periodontal pockets for 30 s. The paper strips were stored in Eppendorf tubes at −80 °C for further elution. Contaminated samples with blood or saliva were discarded. The GCF samples were then eluted using 40 μL Triton ×100 0.01%, complemented with protease inhibitors. Next, tubes were vortexed for 30 s and incubated at 4 °C for 30 min. Following centrifugation at 12,000 rpm for 5 min at 4 °C, the supernatants were collected and stored at −80 °C. The elution procedure was performed twice until obtaining 80 μL/strip.

2.4. NRP-1 Elisa

Maxisorb plates were incubated with 100 μL of capture anti-hNrp-1 antibody diluted in PBS 1× at 1 μg/mL and incubated overnight at 4 °C. After three washes with 200 μL/well of Wash Buffer (0.05% Tween in PBS 1×), the plate was blocked with 200 μL/well of Assay Diluent (10% FBS in PBS 1×) for 1 h at room temperature (RT) and washed as detailed above. The standard curve was prepared using human recombinant NRP-1 (Biolegend, California USA) starting at a concentration of 500 ng/mL, followed by serial dilutions. In parallel, 100 μL of the sample was added to each well, continuing with an incubation period of 2 h at RT. After five washes, 100 μL of biotinylated anti-hNRP-1 was added per well (at one μg/mL in Assay Diluent), followed by an incubation of 1 h at RT. Seven washes followed, after which 100 μL of TMB (Life technologies®, USA) was added to reveal colorimetric changes. The reaction was stopped by adding 2 N H2SO4, and absorbance was measured at 450 nm wavelength using a Tecan absorbance microplate reader (Infinite® 200 PRO NanoQuant, TECAN, USA).

2.5. Statistical analysis

Continuous variables were described with central tendency measures (mean and medians). Shapiro Wilk test was used to evaluate the distribution of the quantitative variables. Mann Whitney test was used to determine the differences between the levels of SNP-1 in patients with or without severe periodontitis (stages III and IV). To determine differences in the concentrations of sNRP-1 comparing among the stages and degree of the disease, we used the Kruskal Wallis test. Spearman test was applied to establish a correlation between the levels of sNRP-1 and the periodontal parameters. For all statistical analyses, the software STATA 14.2 and Prism 8.4.2 were used.

3. Results

Thirty-six patients were recruited, 25 women and 11 men. Their ages ranged between 31 and 53 years, with a median of 49 years old. The distribution by sociodemographic and periodontal status are shown in Table 1.

The concentration of sNRP-1 had a median of 20.085 ng/mL (iqr 12.74) in patients with periodontitis stages I and II and of 38.385 ng/mL (iqr 30.98) ($p = 0.2028$) for patients with severe periodontitis (stages III and IV).

The GCF-sNRP-1 concentration, according to the stage and grade of periodontitis, is presented in Fig. 1 A and B. The concentration of sNRP-1 in GCF of patients in stage II reaches a median of 20.08 ng/mL (iqr 12.74); in contrast, patients with stages III and IV have a median of 40.46 ng/mL (iqr 30.25) and 41.14 ng/mL (iqr 34.65), respectively. Even though we observed a positive correlation between the levels of sNRP-1 and the severity of the disease, this corresponded to a tendency only since the differences were not statistically significant ($p = 0.3607$). Similarly, when we study the relationship between the concentrations of sNRP-1 and periodontitis grade, there were no statistically significant

Table 1
Demographic characteristics of patients with periodontitis stage II and periodontitis stages III – IV.

Demographic Characteristic	Periodontis stage II group (n = 6)	Periodontitis stages III - IV group (n = 30)	p value
Age (yrs):			
Median (iqr)	31 (12)	52.2 (13)	0.0037
Sex:			
Female	7 (28%)	18 (72%)	
Male	0 (0%)	11 (100%)	0.076
Diabetes:			
No	7 (20%)	28 (80%)	
Yes	0 (0%)	1 (100%)	0.9
Smoker:			
No	5 (22.73%)	17 (77.27%)	
Yes	2 (14.29%)	12 (85.71%)	0.681
Body Mass Index			
Median (iqr)	27 (5.1)	27.4 (7.4)	0.7643

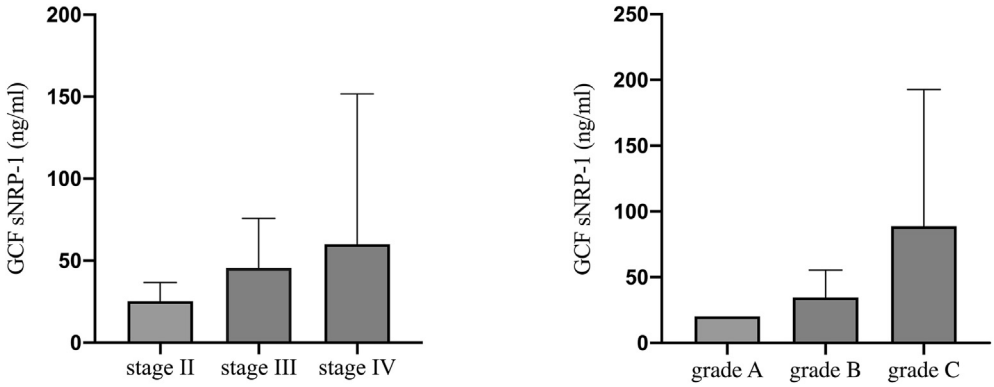

Fig. 1. GCF levels of sNRP-1 according periodontitis stage and grading.

difference (p = 0,1384) between medians of 20.33 ng/mL for grade A (iqr 0), 31.22 ng/mL for grade B (iqr 29.38) and 47.07 ng/mL (iqr 54.3) for grade C.

Finally, we observed a positive correlation between the concentration of sNRP-1 and BOP (Rho: 0.45; p = 0.0048), PISA (Rho: 0.50; p = 0.0019), PD (Rho: 0.3; p = 0.015) and GI (Rho: 0.37; p = 0.02), as displayed in Fig. 2.

4. Discussion

This exploratory cross-sectional study comparatively investigates differences among the levels of sNRP-1 present in GCF from patients with different periodontitis severities. In the present study, we observed a higher concentration of sNRP-1 (almost twice) in patients with stage III and IV periodontitis compared to patients with mild (stage I and II) periodontitis. In light of these findings, we can suggest that sNRP-1 could be involved in periodontitis severity. There is no evidence in the literature to analyze the concentration of sNRP-1 in GCF of patients with periodontitis. Therefore, this study is a pioneer in the association of both variables.

The last periodontal diseases classification proposes to include the utility of different biomarkers for early periodontitis detection. It is possible that soon, it would be feasible to integrate clinical variables, lifestyle habits, and the biomarkers' concentration to classify the severity and future risk of periodontitis and improve the periodontitis' systemic impact on the affected patient.[10]

A biomarker is an objectively measured molecule evaluated as an indicator of normal biological processes.[12] One of these biomarkers is NRP-1, described as a surface receptor on vertebrates, linked to biological processes such as angiogenesis and the immune response.[13] NRP-1 has also been identified as a specific VEGF-A receptor in a spliced form; this binding causes the angiogenic cascade.[13] Moreover, NRP-1 acts as a co-receptor of several molecules, standing out for its role in the formation of the immune synapse between dendritic cells (DC) and T cells, as well as its role in angiogenic processes,[14] which has been used as a biomarker for chronic inflammatory pathologies such as asthma, psoriasis, rheumatoid arthritis and intestinal bowel disease.[15]

Regarding the immune system, NRP-1 is constitutively expressed in the membranes of DC and T cells, regardless of their activation state. In the presence of NRP-1, DC form an immune synapse with T cells.[9] Due to its relationship with inflammatory events, we sought to evaluate the company of sNRP-1 in GCF obtained from periodontitis patients and look for an association between severity and progression rate. Patients affected with severe periodontitis showed an almost 2-fold increment in sNRP-1 than mild to moderate periodontitis patients, suggesting a possible role for sNRP-1 in tissue inflammation observed in severe

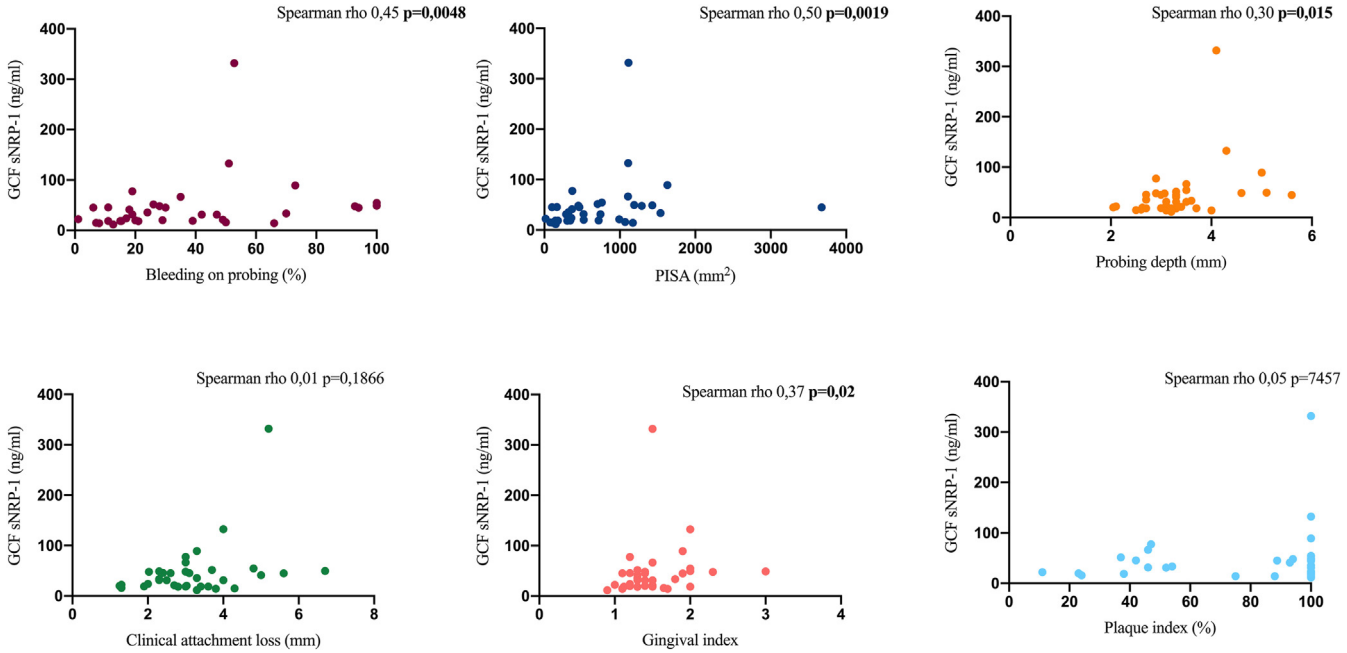

Fig. 2. Spearman correlation between GCF sNRP-1 levels and periodontal parameters.

periodontitis.

When analyzing the GCF samples according to the stages (related to the severity of this disease) and grade (associated with the progression of the disease), we found increased sNRP-1 concentration as the severity of the periodontitis increased, which could indicate more disease severity, more concentration of sNRP-1 is detected. The association between increased concentrations of sNRP-1 with periodontitis severity could be explained by the role of NRP-1 in immunological and angiogenic processes.[8]

In periodontitis, there is a response against periodontopathogen bacteria, which involves innate and adaptive immunity, and could include NRP-1 at DCs and T cells interaction area.[9] An unbalance, or deregulation of this immune response against the periodontal infection provokes excessive tissue damage, with concomitant irreversible destruction of dental support.[16] Therefore, the periodontal destruction could result from an exacerbated angiogenic process, which may positively associate with sNRP-1 and BOP index, suggesting an excessive vascularization of the inflamed periodontal tissue.[13] Furthermore, the higher the levels of sNRP-1, the more significant VEGF binding would be.[17] VEGF is expressed by endothelial cells, gingival epithelial cells, and fibroblasts, and its concentration in GCF of periodontal disease patients is higher than those found in healthy tissue.[17] In this study, we observed a possible positive correlation between the levels of sNRP-1 and the inflammatory periodontal parameters such as BOP, PISA, GI, and PD, which could be linked to an increased tissue vascularization triggered or mediated by sNRP-1. However, further analysis is required to conclude this observation fully.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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