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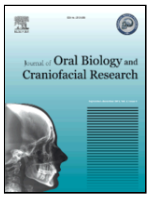

Soluble neuropilin-1 in gingival crevicular fluid is associated with rheumatoid arthritis: An exploratory case-control study

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ABSTRACT

Background: To explore the soluble Neuropilin-1 (sNRP-1) concentrations in gingival crevicular fluid (GCF) and the periodontal clinical status of patients with Rheumatoid Arthritis (RA).
Materials and methods: We conducted an exploratory study with 40 study participants, 20 with RA, and 20 healthy controls. Clinical and periodontal data were recorded, and GCF samples were obtained. sNRP-1 levels in GCF were determined by ELISA assay. Descriptive statistics, Mann–Whitney *U* test, Unpaired *t*-test, logistic regression model, and Area Under Receiver Operating Characteristic Curve (AUC-ROC) were made to explore the diagnostic performance accuracy.
Results: RA patients had significantly higher levels of sNRP-1 in GCF ($p = 0.0447$). The median levels of GCF-sNRP-1 were 208.85 pg/μl (IQR 131.03) in the RA group compared to 81.46 pg/μl (IQR 163.73) in the control group. We observed an association between the GCF-sNRP-1 concentrations and the RA diagnosis (OR:1.009; CI 1.00–1.001; $p = 0.047$). The diagnosis of chronic periodontitis was also associated with RA (OR: 6.9; CI 1.52–31.37; $p = 0.012$). Moreover, the AUC-ROC of GCF-sNRP-1 concentrations combined with periodontal clinical parameters such as periodontal probing depth and periodontal inflamed surface area was 0.80.
Conclusion: This exploratory case-control study shows that RA patients had significantly higher levels of sNRP-1 in GCF. New longitudinal studies are necessary to evaluate the role of NRP-1 in periodontal tissues and consider it an oral biomarker with clinical value in RA.

1. Introduction

Rheumatoid Arthritis (RA) is a systemic autoimmune disease characterized by the joints' chronic inflammation, even producing its destruction in the long term. Part of its etiology is explained by the infiltration and activation of immune cells in the synovial fluid, affecting the synovial membrane. Patients who suffer from RA have chronic pain and functional disabilities that increase over time, and, without treatment, their life expectancy decreases from 10 to 3 years.[1–3] The prevalence of RA is around 1% in most of the European population. It varies between 0.4 and 1% in Latin America, being more common in females (ratio of 8:1).[3–5] In the United States, the prevalence of RA ranged from 0.41 to 54% from 2004 to 2014,[6] and in other countries like India, the prevalence ranged from 0.28 to 0.7%.[7]

The activation of a chronic inflammatory process within the affected joints promotes hyperplasia of the synovial membrane, angiogenesis, and tissue destruction.[2] The accumulation of synovial stromal fibroblast-like synoviocytes (FLS) is also a hallmark of RA, and they contribute to the chronic inflammatory process and angiogenesis observed in the synovial hyperplasia.[8] Previous mechanistic studies have evaluated the role of Neuropilin (NRP) in AR study participants[8,9] and the potential effects of anti-NRP-1 treatment on T cell differentiation and angiogenesis on experimental AR models.[10,11] NRPs are non-tyrosine kinase cell surface/transmembrane glycoproteins expressed in all vertebrates and widely conserved across species. These proteins are involved in angiogenesis, immunity, migration, recruitment, communication between different immune cells, and pathological conditions, such as cancer and autoimmune diseases.[12] NRPs are also detected in osteoclasts and osteoblasts, where they regulate bone homeostasis.[13] Inflammatory cells like macrophages, dendritic cells, and regulatory T-cells express NRP-1, characterized mainly in T cells.[13] NRP-1 also serves as a receptor of semaphorin-3A (SEMA3A), micro-RNAs, and the vascular endothelial growth factor (VEGF).[14,15] Furthermore, other reports considered soluble NRP-1 (sNRP-1) a VEGF antagonist because it inhibits the downstream effects of VEFG signaling and has shown an anti-angiogenic impact.[13,16]

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NRP-1 expression increases in immune cells involved in the RA pathogenesis, and the VEGF expression (one of its ligands) are also elevated in RA joints. Furthermore, the levels of VEGF in synovial tissues correlate with the degree of joint destruction. Besides, RA patients develop a potent angiogenic activity during the early stages of the disease. Moreover, VEGF's blockade by binding to NRP-1, using an anti-NRP-1 peptide, diminishes experimental RA's severity.[10] Alternatively, the downregulation of NRP-1 expression with siRNA led to synoviocyte apoptosis, suggesting that NRP-1/VEGF binding is necessary for the RA joint damage.[17]

On the other hand, some epidemiological studies support the association between RA and periodontitis.[18–24] A systematic review and meta-analysis reported a prevalence of periodontitis in RA patients ranging from 15.5% to 100%, with an OR for periodontitis in RA study participants of 4.68 (95% CI: 3.11–7.05). The authors suggested that RA was associated with an increased overall risk of periodontitis.[25] This link is mainly mediated by the presence of *Porphyromonas gingivalis* (*P.gingivalis*), a cornerstone pathogen in the pathogenesis of periodontitis, which can also be isolated in inflamed joints of RA patients and produces the enzyme peptidyl arginine deiminase (PPAD), which converts arginine residues into citrulline.[21,26] This protein citrullination process leads to the activation of anti-citrullinated protein antibodies (ACPAs).[27] The immune response against citrullinated proteins evoked by *P.gingivalis* during RA development in a susceptible host is the primary mechanism involved in the association between RA and periodontitis.[18,20,28]

Moreover, pro-inflammatory cytokines such as IL-1, IL-6, and TNF have shared molecules found in RA and periodontitis patients and are involved in tissue destruction.[29] Furthermore, the expression of NRP-1 has also been involved in RA pathogenesis, mediated explicitly by NRP-1 + T regulatory cells.[30] Our research team recently found increased levels of sNRP-1 in GCF of patients with severe periodontitis, and the concentration was correlated with periodontal clinical inflammatory manifestations. It probably could be involved in pro-inflammatory and angiogenic mechanisms observed in periodontitis.[31]

Considering the chronic inflammatory nature of RA and periodontitis and the similarity in both diseases' pathogenesis, we hypothesize that sNRP-1 levels could be increased in the gingival crevicular fluid (GCF) of RA patients, which could be involved in the severity of periodontitis and periodontal clinical inflammatory parameters in these patients. Thus, we also explore the sNRP-1 levels in GCF as a potential oral biomarker of RA and the relationship between RA and periodontitis.

2. Material and methods

2.1. Study design

We performed an exploratory case-control study in the Health Care Centre of Universidad de Los Andes and Universidad de La Frontera, Chile. Enrolment, physical and periodontal data were recorded from 40 study participants from both genders, aged between 20 and 83. Of them, 20 patients were diagnosed with RA, and 20 healthy participants were included as controls. A complete full-mouth periodontal examination was conducted by a periodontist, including pocket probing depth (PPD), clinical attachment loss (CAL), bleeding on probing (BOP), periodontal inflamed surface area (PISA),[32] and plaque index (PI). The study's exclusion criteria included patients with fewer than 16 teeth, diagnosed with chronic systemic inflammatory, metabolic, or autoimmune diseases, pregnancy or lactation, use of antibiotics during the last three months, or periodontal therapy during the previous six months before the recruitment of orthodontics treatment. After a complete periodontal examination, GCF samples were collected. The present study was approved by the Universidad de la Frontera (Nº 024/17) Ethics Committee. All patients participating in the study consent to participate by signing the appropriate informed consent. We declare that the study was performed in full accordance with the Declaration of Helsinki.

2.2. Diagnostic criteria

RA diagnosis was established using the EULAR/ACR criteria,[33] which considers the number of sites and joints involved, serological abnormalities, increased acute-phase molecules, and symptoms. Chronic periodontitis was diagnosed when four or more teeth showed one or more sites with a probing pocket depth (PPD) of 4 mm or higher, as well as if they had a clinical attachment loss (CAL) of 3 mm or higher at the same site, inflammation and bleeding on probing (BOP).[34]

2.3. Gingival crevicular fluid samples: collection and elution

GCF samples were collected using PeriopaperTM strips (Oralflow, Smithtown, NY, USA). All samples were collected from the mesiobuccal site of the sulcus/pocket. Four periodontal pockets (1 per quadrant) PPD $>$ 3 mm and CAL $\geq$ 3 mm were selected in periodontitis patients. In the healthy/gingivitis group, four shallow periodontal sulcus sites (1 per quadrant) PPD $<$ 3 mm, without CAL and positive BOP until 10%. After the tooth was isolated with a cotton roll, the supragingival plaque was removed with curettes (Hu Friedy, Gracey, IL, USA) without touching the marginal gingiva. The gingival sulcus was then gently dried with a syringe. The strips were placed into the sulcus/pocket until mild resistance was sensed and left in place for the 30s. Strips contaminated with saliva or blood were excluded from the study. After GCF collection, the strips were placed in Eppendorf vials containing 100 μl of phosphate-buffered saline (PBS) with 0.05% Tween-20 (PBS-T). Immediately, GCF was extracted by centrifugation at 12,000 g for 5 min at 4 °C. The elution procedure was repeated, and both eluates were pooled and stored at −80 °C until analysis.

2.4. Enzyme-linked ImmunoSorbent assay for sNRP-1

Maxisorb plates were incubated with 100 μL of capture anti-hNrp-1 antibody diluted in PBS 1X 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 1X), the plate was blocked with 200 μL/well of Assay Diluent (10% FBS in PBS 1X) for 1 h at room temperature (RT) and washed as detailed above. Next, the standard curve was prepared by using human recombinant NRP-1 (RyD 3870-N1-025), 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 (Biolegend, California USA) was added per well (at 1 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 2N H2SO4, and absorbance was measured at 450 nm wavelength using a Tecan absorbance microplate reader (Infinite® 200 PRO NanoQuant, TECAN, USA). The concentrations of sNRP-1 in GCF samples were determined by interpolation from the standard curve. Additionally, the laboratory personnel performing the assays were blinded to the clinical information.

2.5. Statistical analysis

Data normality was tested using the Shapiro-Wilk test. PPD, CAL, and age were normally distributed. Concentrations of sNRP-1 in GCF, BOP, PI, and PISA were not normally distributed; therefore, parametric and non-parametric tests were used. The unpaired *t*-test test and Mann–Whitney *U* test were used for comparisons of continuous variables. The evaluation of the diagnostic accuracy of PISA and sNRP-1 was performed through the construction of ROC curves by calculating the area under the curve (AUC). The optimal cut-off points to estimate Youden's Index altogether assessed the highest sensitivity and specificity. The statistical analysis was performed using STATA 14.2 (Texas, USA). A p-value <0.05 was considered statistically significant.

3. Results

From the forty study participants recruited in the present pilot study, 10 (25%) corresponded to females and 30 (75%) to males. The mean age was 48.74 years (SD 15.73). Three (21.43%) participants of the control group and seventeen (65.38%) of the RA patients were diagnosed with chronic periodontitis. Periodontal clinical parameters such as PPD and PISA were significantly increased in the RA patients than in the control group (p-value < 0.05). The distribution of the demographic and periodontal parameters is presented in Table 1.

sNRP-1 was detected in 26 (65%) patients. Among them, 14 (53.85%) were RA patients, and 12 (46.13%) were from the control group. The observed GCF-sNRP-1 concentrations were 208.85 pg/μl (IQR 131.03) in RA group compared with 81.46 pg/μl (IQR 163.73) in the control group, suggesting that GCF-sNRP-1 concentrations are increased in RA patients (p-value = 0.0447) (Fig. 1A). The regression logistic model shows an association between the GCF-sNRP-1 concentrations and the diagnosis of RA (OR:1.009; CI 1.00–1.001; p-value = 0.047). Furthermore, the diagnosis of periodontitis was associated with the RA condition in this group of participants (OR: 6.9; CI 1.52–31.37; p-value = 0.012) (Table 2). The observed GCF-sNRP-1 concentration were 188.19 pg/ul (IQR 116.16) in periodontitis patients compared to 91.83 pg/ul (IQR 117.02) in the healthy/gingivitis patients (p-value = 0.28) (Fig. 1B).

The area under the ROC curve observed for GCF-sNRP-1 concentrations for the RA diagnosis was 0.64 (Fig. 2). The clinical periodontal parameters that showed acceptable discrimination (AUC ≥70) by themselves for RA diagnosis were PPD and PISA. Then, a new ROC curve was generated, including the GCF-sNRP-1 concentrations, PPD, and PISA. The observed performance under the new ROC curve was 0.80 (Fig. 2).

Table 1
Description of the demographic and periodontal clinical parameters of study participants included in the study according to RA status.

Variable	Control group (n = 20)	RA group (n = 20)	*p*-value
Age	36.5 (17.5)	53 (27.5)	***0.0005****
Sex (male)	13 (65%)	17 (85%)	*0.144*
Smoking	6 (30%)	5 (25%)	*0.723*
Body mass index (BMI)	25.09 (5.16)	26 (4.54)	*0.185*
Bleeding on probing (BOP) (%)	19.25 (10.8)	23.36 (42.90)	*0.7455*
Plaque Index (PI) (%)	80.75 (10.8)	76.63 (42.9)	*0.7451*
Periodontal probing depth (PPD) (mm)	2.5 (0.65)	3.25 [1]	***0.0082****
Clinical attachment loss (CAL) (mm)	2.7 (1.5)	3.4 (1.15)	*0.1224*
Periodontal inflamed surface area (PISA) (mm [2])	283.2 (849.88)	946.17 (1226.44)	***0.0038****

***(Bold)** significance p-value (<0.05, *Mann Whitney Test*). Results are expressed in median values (P50) with interquartile range.

4. Discussion

The results from this exploratory case-control study suggest that RA patients had significantly higher levels of sNRP-1 in GCF compared with healthy controls (p-value = 0.0447). Also, the logistic regression model shows an association between the GCF-sNRP-1 concentration and the RA diagnosis. This is the first study that assesses sNRP-1 levels in GCF from patients with RA to the best of our knowledge. Additionally, the findings of this study confirm an association between periodontitis and RA.

RA is a systemic autoimmune and inflammatory disease that causes joints inflammation and tissular destruction, accompanied by pain and functional disability of the patients.[29] At the same time, periodontitis is characterized by chronic inflammation of supporting periodontal tissues, loss of clinical attachment, and severe cases, including some teeth.[35] Periodontitis is caused by characteristic pathogens like *P. gingivalis*, *Treponema denticola,* and *Fusobacterium nucleatum* in a susceptible host, triggering local environmental changes that lead to a dysbiotic relationship with the host, favoring the proliferation of subgingival pathobionts pathogens. These bacteria can evade the host's immune response and attract an increment in the cell infiltrate with an increased synthesis of pro-inflammatory cytokines and mediators.[36,37] Both diseases, RA and periodontitis, have in common the cellular infiltrate and the release of a significant amount of inflammatory molecules such interleukin −4, −6, -1B -10, prostaglandin E2 and the Tumoral Necrosis Factor-alpha (TNF-α), among others.[38,39]

Furthermore, a recent study confirms that most RA patients had moderate/severe periodontitis, which agrees with the present results. Besides, the periodontitis severity was significantly associated with the ACPA positivity (86% versus 50%), and altered subgingival microbial profile, and increased levels of systemic and oral pro-inflammatory mediators such as sCD30/TNFRSF8, IFN-α2, IL-19, IL-26, MMP-1, gp130/sIL-6Rβ, and sTNF-R1.[40] Aside from that, it has been reported that antibodies to *P. gingivalis* are increased in RA patients. RA patients with severe periodontitis present a more robust antibody response against *P. gingivalis* than healthy controls.[41] The primary role of *P. gingivalis* in the pathogenesis of RA is their involvement in the citrullination of peptides mediated by peptidyl arginine deiminase (PAD), an enzyme secreted by immune cells such as T and B lymphocytes, neutrophils, monocytes, and macrophages, which leads to the production of anti-CCP antibodies.[42] Citrullination is a post-translational modification of the amino acid arginine into citrulline mediated by PAD.[42] *P. gingivalis* also express the PAD enzyme, promoting the ACPA formation, thus playing an essential role in RA's pathogenesis.[43]

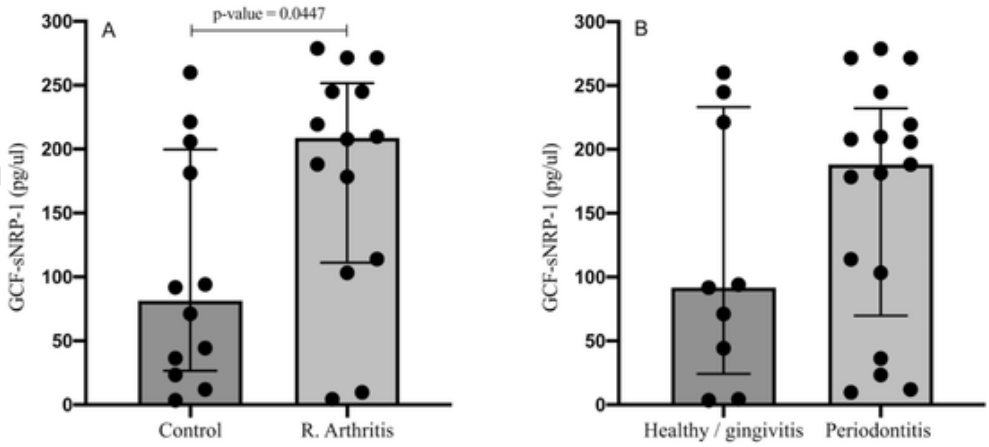

Fig. 1. sNRP-1 concentrations in gingival crevicular fluid (GCF) according to RA diagnosis (A) and Periodontitis diagnosis (B).

Table 2
Periodontitis diagnosis and RA association model (Simple logistic regression).

Parameter	OR	IC 95%	*p - value*
Sex (male)	3.05	0.65–14.13	*0.154*
Age	1.09	1.02–1.16	***0.004****
Periodontitis	6.92	1.52–31.37	***0.012****
Periodontitis + Age	2.43	0.41–14.15	*0.322*

OR: Odd ratio, IC: Interval confidence and ***(Bold)** significance p-value < 0.05.

NRP-1 is a transmembrane glycoprotein that acts as a no tyrosine kinase receptor for $VEGF_{165}$.[44] It has been linked to biological processes such as angiogenesis and the immune response. It has an essential function as a co-receptor of several molecules, standing out for its role in forming the immune synapse between dendritic cells and T cells and its role in angiogenic processes.[45] Angiogenesis is a prevalent finding in RA, particularly during the early stages of the disease where these new capillary blood vessels promote a chronic inflammation status by stimulating the migration of inflammatory cells to the sites of synovitis, as well as by providing nutrients and oxygen to the inflamed tissues.[10] Also, NRP-1 has been identified as a specific VEGF-A receptor in a spliced form, causing the angiogenic cascade's initiation upon receptor binding.[46] Moreover, in RA fibroblast-like synoviocytes (FLS), the primary binding site of $VEGF_{165}$ is NRP-1, and the binding of $VEGF_{165}$ to NRP-1 protects rheumatoid synoviocytes from apoptotic death by regulating the expression of Bcl-2 and translocation of Bax, which contribute to the synovial hyperplasia that plays an essential role in RA pathogenesis.[17] Similarly, but needing further clarification, NRP-1 could be involved in the more significant periodontal inflammation observed in RA patients with periodontitis.

Furthermore, NRP-1 has also been used as a biomarker for chronic inflammatory disorders such as asthma, psoriasis, RA, and intestinal bowel disease.[47] Considering that synoviocyte adhesion and migration are critical for synoviocyte activation/survival and joint destruction, it has been proposed that anti-NRP-1 could regulate the RA inflammation.[48,49] The use of biomarkers for RA diagnosis includes inflammatory molecules and immunity, such as C-reactive protein (CRP), rheumatoid factor (RF), and antibodies against citrullinated proteins (ACPA).[50,51] Both RF and ACPA can be used for RA detection before clinical onset. However, their sensibility and specificity could be increased if novel biomarkers are incorporated into a RA activity prediction algorithm.[52]

In summary, our findings provide new insights into the participation role of sNRP-1 in periodontal inflammation of RA patients. However, the present results must be interpreted with caution due to the limited sample and the study's exploratory case-control nature. The underlying mechanisms involved between sNRP-1 and the periodontal and RA inflamed tissues have not been explored and are still unclear. Thus, future studies need to include a prospective cohort design oriented to clarify the specific role of sNRP-1 in periodontal inflamed tissues by RA status and disease activity and their potential as prognostic/monitoring biomarkers in oral fluids of the activity of RA patients. It would be interesting to study gingival tissue to determine which cells express the NRP-1 receptor in their membrane and associate it with angiogenic and immunological processes.

Despite this study's limitations, it is the first to show that RA patients had significantly higher levels of sNRP-1 in GCF. Likewise, we confirm the association between periodontitis and RA several times established in the literature. Furthermore, more research is required to evaluate the role of NRP-1 in periodontal tissues and consider it a plausible oral biomarker for RA monitoring.

Declaration of competing interest

The authors of this study declare that they have no conflicts of interest. This study was financed by internal funds from Universidad de los Andes (Santiago, Chile) and Universidad de la Frontera (Temuco, Chile).

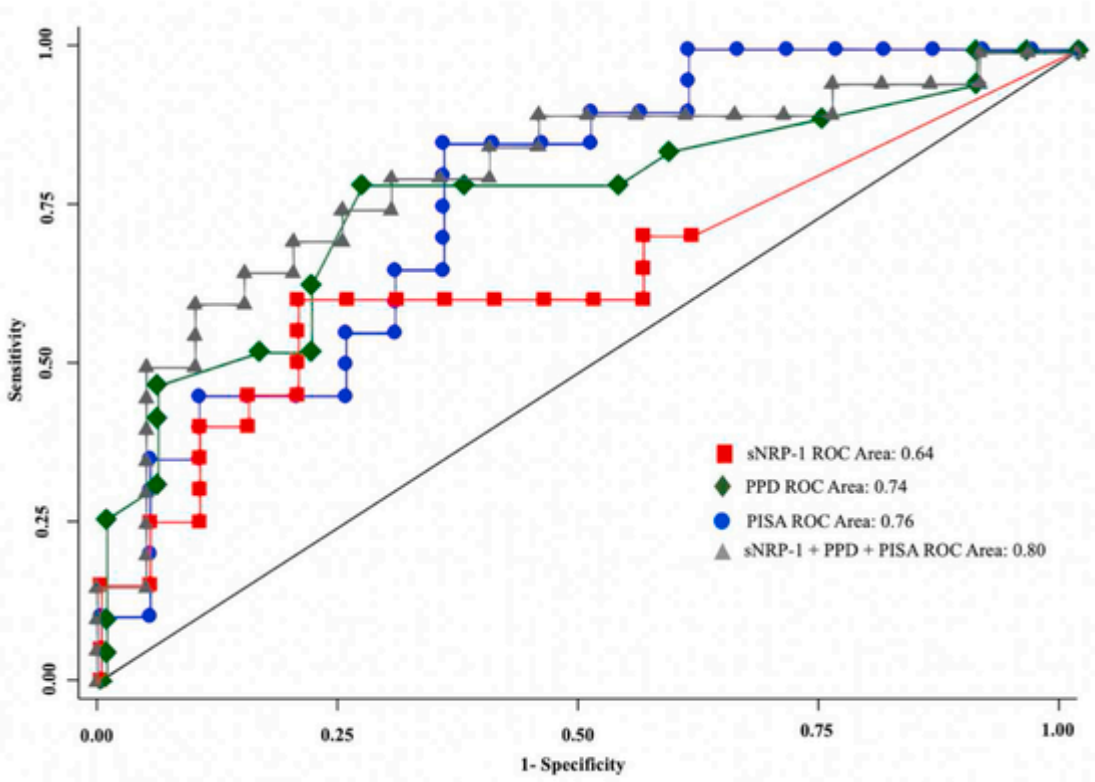

Fig. 2. Receiver Operating Characteristic curve (ROC-curve) of GCF-sNRP-1 concentrations, periodontal probing depth (PPD) and periodontal inflamed surface (PISA) versus RA diagnosis.

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