Lymphocyte B and Th17 chemotactic cytokine levels in peri-implant crevicular fluid of patients with healthy, peri-mucositis, and peri-implantitis implants.

Niveles de citoquinas quimiotácticas de linfocitos B y Th17 en el fluido crevicular periimplantario de pacientes con implantes sanos, perimucositis y periimplantitis.

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Abstract: Peri-implantitis is one of the leading causes of implant failure and loss, and its early diagnosis is not currently feasible due to the low sensitivity of currents methods. In the current exploratory cross-sectional study, we explored the diagnostic potential of lymphocyte B and Th17-chemotactic cytokine levels in peri-implant crevicular fluid (PICF) in 54 patients with healthy, peri-mucositis, or peri-implantitis implants. Peri-implant crevicular fluid was collected, and the levels of the molecules under study were quantified by Luminex assay. The concentrations of CCL-20 MIP-3 alpha, BAFF/BLYS, RANKL and OPG concentration in PICF were analyzed in the context of patient and clinical variables (smoking status, history of periodontitis, periodontal diagnosis, implant survival, suppuration, bleeding on probing, periodontal probing depth, clinical attachment level, mean of implant probing depth, and plaque index). Patients with peri-implantitis, appear to have an overregulation of the RANKL/BAFF-BLyS axis. This phenomenon needs to be investigated in depth in further studies with a larger sample size.

Keywords: *Peri-implantitis; mucositis; chemokine ccl20; RANK ligand; enzyme-linked immunosorbent assay; gingival crevicular fluid.*

Resumen: La periimplantitis es una de las principales causas de falla y pérdida del implante, y su diagnóstico temprano no es factible debido a la baja sensibilidad de los métodos actuales. En este estudio transversal exploratorio, se estudió el potencial diagnóstico de los niveles de citocinas quimiotácticas de linfocitos B y Th17 en el líquido crevicular periimplantario (LCPI) en 54 pacientes con implantes sanos, peri-mucositis o periimplantitis. Se recogió líquido crevicular periimplantario y se cuantificaron los niveles de las moléculas estudiadas mediante Luminex assay. Las concentraciones de CCL-20 MIP-3 alfa, BAFF/BLYS, RANKL y la concentración de OPG en LCPI se analizaron en el contexto de las variables clínicas y del paciente (tabaquismo, antecedentes de periodontitis, diagnóstico periodontal, supervivencia del implante, supuración, sangrado al sondaje, profundidad de sondeo periodontal, nivel de inserción clínica, media de la profundidad de sondeo del implante e índice de placa). Los pacientes con periimplantitis parecen tener una sobrerregulación del eje RANKL/BAFF-BLyS. Este fenómeno debe investigarse en profundidad en futuros estudios con un tamaño de muestra mayor.

Palabra Clave: *Periimplantitis; mucositis; quimiocina CCL20; ligando RANK; ensayo de inmunoadsorción enzimática; líquido del surco gingival.*

INTRODUCTION.

Peri-implantitis (PI) is one of the leading causes of implant failure and loss. It initiates with an imbalance between the bacterial biofilm and the host response at the implant surrounding mucosa, which triggers an immune-inflammatory reaction known as peri-mucositis and peri-implantitis.[1]

The risk factors for peri-implantitis are previous history of periodontitis, smoking, diabetes, poor plaque control, and lack of regular maintenance therapy.[2] In clinics, the diagnosis of peri-implant mucositis and peri-implantitis is mostly carried out by clinical and radiographic examination.

Clinical symptoms can include bleeding on probing (BOP), increased probing depth (PD) and suppuration. Radiographic examination aids by determining bone loss. However, the sensitivity of these methods is quite low, with detection only possible once tissue damage has already occurred. Therefore, efforts are being placed on developing effective complementary diagnostic tools to aid the clinical and radiographic screening. Overall, early and precise diagnosis of peri-implant diseases remains difficult and even more, the treatment protocol is not predictable, is still under revision, and not universally established.

One of the current approaches is the analysis of biomarkers in the peri-implant crevicular fluid (PICF). Biomarkers are characteristics that can be objectively measured and evaluated, which are indicators of a normal or pathological biological process, and that can be quantified with reproducibility. In recent years, several relevant biomarkers for PI have been described including interleukin-1 beta (IL-1β), plasma tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-8 (MMP-8), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG).[3,4]

These molecules and others, such as lymphocyte B and T chemotactic cytokine (CCL-20 and BAFF/BLYS), which are the main producers of RANKL in PI inflamed tissues, have important roles in modulating inflammatory responses, and their expression in the PICF promotes osteoclastogenesis,[3,4] which is one of the main characteristic of PI.

Based on this approach, in which we aim to develop tailored strategies for the early diagnosis and treatment of peri-implant diseases, we need to take advantage of oral fluids as a source of biomarkers to detect the risk of peri-implantitis during the early and asymptomatic stages. Furthermore, the study of biomarkers in oral fluids could represent a promising part of new predictive and diagnostic tools that combine peri-implant parameters, genetics, clinical data, and risk behaviors of the patient.

The present study aimed to explore the diagnostic potential of lymphocyte B and Th17-chemotactic cytokine levels in peri-implant crevicular fluid (PICF) of patients with healthy, peri-mucositis, and peri-implantitis implants.

MATERIALS AND METHODS.

Study design

An exploratory cross-sectional study was conducted in the Health Care Centre of the Universidad de Los Andes and the Universidad de la Frontera, in Santiago and Temuco, respectively, Chile.

Fifty-four patients were enrolled, and their clinical, physical and periodontal data was recorded. Among the recruited patients, 17 were diagnosed as healthy implants (H), 19 patients with implants with peri-implant mucositis (PM) and 18 with peri-implantitis (PI).

All subjects were systemically healthy (both sexes) and were aged between 30-78 years old. Complete full-mouth periodontal examinations were performed by a periodontist, including bleeding on probing, peri-implant probing depth, clinical attachment loss and plaque index. Exclusion parameters for the study were: chronic inflammatory diseases (diabetes, cardiovascular, chronic inflammatory, autoimmune and infectious diseases), systemic or topical antimicrobial/anti-inflammatory therapy for the previous three months. After a periodontal and peri-implant exam, PICF samples were collected.

All clinical data for the study were recorded and after, the patients were derived to peri-implant therapy. This study was approved by the Universidad de La Frontera Scientific Ethics Committee. All patients participating in the study consented to participate by signing the appropriate informed consent form.

The studied variables were smoking status, history of periodontitis, periodontal diagnosis, implant survival, suppuration, bleeding on probing, periodontal probing depth, clinical attachment level, mean of implant probing depth, plaque index, CCL-20 MIP-3 alpha, BAFF/BLYS, RANKL and OPG levels in PICF.

Diagnostic Criteria

Peri-implant health was diagnosed as absence of swelling, bleeding on probing, inflammation and suppuration; besides the absence of increased probing depth and the absence of radiographic bone loss.[5]

On the other hand, peri-mucositis was described as inflammation of the peri-implant soft tissues, without bone loss, but with bleeding on probing, swelling, and suppuration in some cases.[6]

Finally, peri-implantitis was characterized by the presence of inflammation, as in peri-mucositis, but with the presence of progressive bone loss.[1-7]

Peri-implant crevicular fluid

Collection and elution of PICF samples: Samples were collected using Periopaper™ strips (Oraflow, Smithtown, NY, USA). Briefly, the supragingival plaque was removed using curettes without contacting the marginal gingiva, and the gingival sulcus/pocket was then dried gently with an air syringe. Strips were inserted 3-5mm into the sulci/pockets for 30 seconds. Strips contaminated by saliva and/or blood were discarded.

Samples were then stored in 1.5mL tubes at -80°C until elution. For elution of PICF, four strips per implant were placed into a 1.5ml tube containing 160 µL of phosphate buffer saline (PBS) (Corning, Mediatech Inc, NY, USA) and protease inhibitor cocktail (EDTA Complete™, mini, EDTA-free Protease Inhibitor Cocktail, Roche, USA). Tubes were vortexed and incubated on ice for 30 min, and then centrifuged at 12,000 x g for 5 min at 4°C.

The eluate was collected and placed on ice. The elution procedure was repeated and both eluates were pooled and stored at -80°C until analysis.

Luminex Assay

CCL-20 MIP-3 alpha, BAFF/BLYS, RANKL and OPG concentrations in PICF samples were quantified using a custom-designed multiplex Luminex assay kit. Samples were analyzed using the multiplex assay, according to manufacturer instructions.

All samples were analyzed by duplicate. Briefly, the re-suspended microsphere cocktail (50 µl) was added to each well of a 96-well black plate. PICF eluate (50 µl) was added to each well.

The plates were carefully covered with an aluminum foil plate sealer and incubated at room temperature for 2 hr in a horizontal orbital plate-agitator at 800 ± 50 rpm.

Plates then were placed in a specially designed magnet plate holder for 1 min and the liquid was discarded. Each well was washed with 100 µl of wash buffer for 1 min in the magnet plate and then the liquid was discarded again. Biotin antibody cocktail (50 µl) was added to each well, the plate was sealed and incubated at room temperature with agitation for 1 hr.

Each well was then washed with wash buffer as previously described and diluted Streptavidin-PE (50 µl) was added. The plate was incubated for 30 min, as previously described. A new wash was performed, followed by a re-suspension of the microspheres in 100 µl of wash buffer and incubation for 2 min.

Finally, samples were detected using MAGPIX® System. The final concentration of the samples was calculated using a Milliplex Analyst (version 5.1; Merck KGaA, Darmstadt, Germany)

Statistical Analysis

By implant status, categorical variables were described through frequencies and percentages, and continuous variables were described through the median and interquartile range. Comparisons between biomarkers levels were explored using the Kruskal-Wallis test .

A p-value<= 0.05 was considered statically significant. The analysis was performed using STATA software (version 15.1; StataCorp, College Station, Texas, US).

RESULTS.

A total of 54 patients were recruited in the present pilot study, 17 healthy subjects, 19 patients with peri-mucositis, and 18 with peri-implantitis.

Age, sex, smoking status, mean of periodontal probing depth, mean of clinical attachment level and suppuration did not differ significantly between healthy (H), peri-mucositis (PM) and peri-implantitis (PI) patients. Previous history of periodontitis (p=0.045), bleeding on probing (BOP) (p=0.005), plaque index (p<0.0001) and implant probing depth (p=0.0002), were significantly higher in PI patients compared to H implants.

The median CCL20/MIP-3 alpha-PICF concentration was 57.29 pg/ml (33.24–103.84) in H implants, 73.21 pg/ml (27.68–115.41) in PM implants and 48.845 (29.16–83.82) in PI implants (p=0.802). RANKL-PICF concentration was 606.78 pg/ml (584.06–612.46) in H implants, 595.42 pg/ml (572.7–595.42) in PM and 652.2 pg/ml (629.5–765.62) in PI implants (p=0.001,

H *versus* PI *p*=0.003 and M *versus* PI *p*=0.0002, respectively). OPG-PICF concentration was 1701 pg/ml (1361–2301) in H implants, 1701 pg/ml (1168–2194) in PM implants and 2023.5 pg/ml (1462–3231) in PI implants (p=0.450). The median of the RANKL/OPG ratio was 0.29 (0.26–0.43) in H implants, 0.37 (0.24–0.58) in PM and 0.31 (0.21–0.56) in PI implants (p=0.770).

Figure 1. Concentrations of cytokines in peri-implant crevicular fluid (PICF) samples from healthy, mucositis and periimplantitis patients
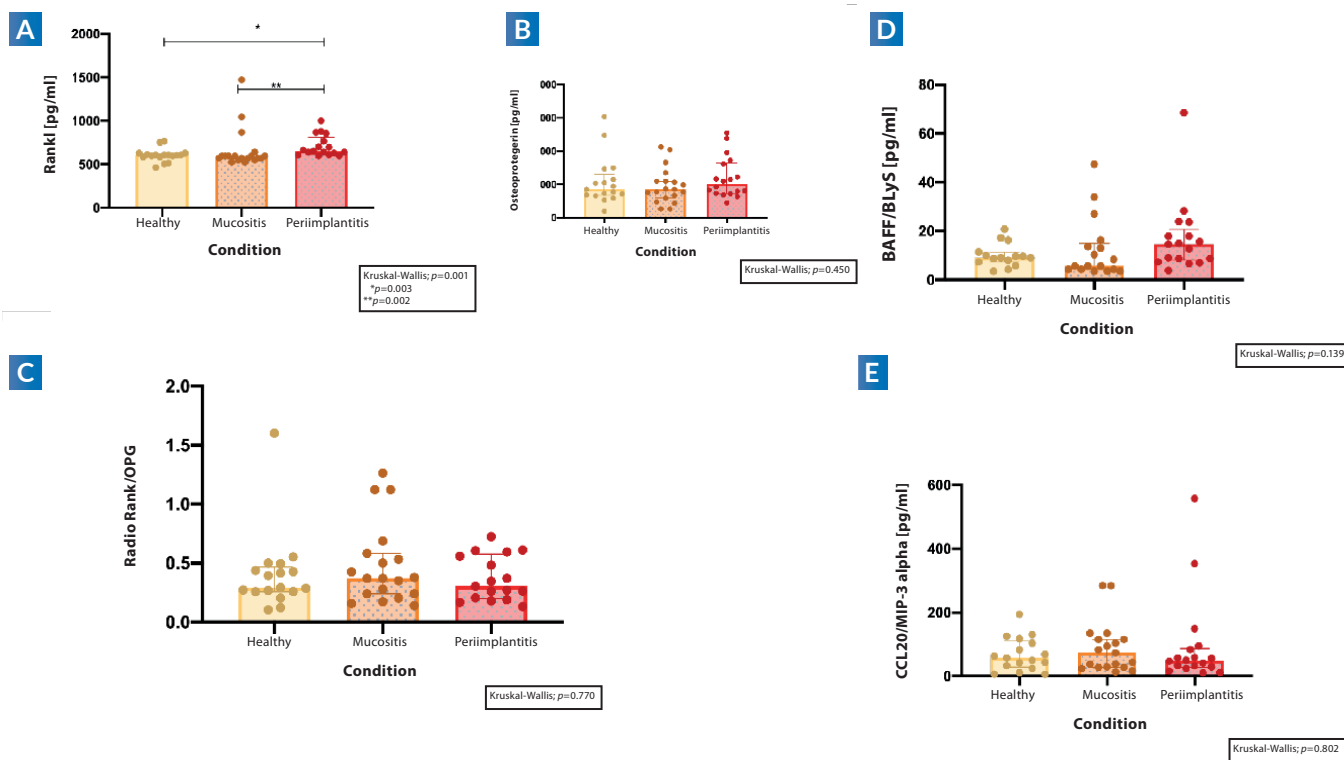
A: RANKL. **B:** OPG. **C:** RANKL/OPG ratio. **D:** BAFF/BLyS. E: CCL20/MIP-3.

DISCUSSION.

A biomarker or "biological marker", refers to a broad subcategory of medical signs, *i.e.* objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly, like signs of the disease (objective characteristic) and levels of different molecules, that are actually studied based on multi-omics analyses.[8]

After many years, biomarkers were introduced as diagnostic criteria in the new classification of periodontal and peri-implant conditions. However, the standardization and processing of sample collection is still necessary. The poor clinical study designs and the lack of standards for specimen collection (for example, a good clinical definition of peri-implantitis) are challenges for the development of new biomarker targets.

RANKL is one of the most widely described bone metabolism related proteins with potential use as a marker for bone destruction in periodontal and peri-implant diseases.[9,10] The results of this study are in line with the available evidence showing high levels of RANKL in peri-implant patients in comparison with healthy subjects.[11]

However, in the case of OPG, due to varying results, additional evidence is needed to validate this potential link. It is important to considerer the time of evolution of the peri-implant disease. Some studies have found higher levels of RANKL in peri-implant mucositis than in peri-implantitis, probably due to the primary function of this molecule in the initiation of osteoclastogenesis compared to the levels in established bone lesions such as peri-implantitis, since the maintenance of activated

osteoclasts represents its secondary function.[12] In this context, the design of clusters of biomarkers could be necessary to determine the presence of the pathology, using biomarkers derived from the peri-implant biofilm, and from the destruction process of soft and hard tissues.[13]

According to our results, no statistically significant differences were found in the levels of CCL20/MIP-3 in PICF. More studies with a greater number of samples are required to confirm the biological plausibility of this molecule as a biomarker of peri-implant disease. It could be interesting to analyze and correlate the levels of CCL20/MIP-3 with the type of cells present in the tissue, considering the CCL20/MIP-3 relation with Th17 cells migration, and the capacity of some periodontal pathogens to induce the overexpression of this molecule in different cell types.[14,15]

Increased serum levels of BAFF and TNF-a have previously been reported in patients with rheumatoid arthritis compared to systemically healthy women with periodontal disease, and BAFF was also correlated with levels of RANKL in serum and PICF ($p<0.05$).[16]

These same authors suggest that the long use of inflammatory drug in patients with rheumatoid arthritis and osteoporosis, together with increased TNF-family cytokines might suggest that these patients are more likely to overproduce these inflammatory mediators. However, whether this results from greater disease activity or contribute to greater disease activity remains moot.[16]

Studies conducted in ligation-induced periodontitis in mice with B-cell deficiency compared to wild type controls, reported that both BAFF and APRIL support the survival of B-cells expressing RANKL, thus contributing to bone loss in the course of chronic periodontitis.[17] In this same context, in a study conducted in a cohort of patients with chronic and aggressive periodontitis, increased levels of BAFF in serum and saliva were found versus periodontally healthy patients.[18] However, results of BAFF expression in peri-implant diseases in humans have not yet been reported. The preliminary results of this study suggest that increased levels BAFF in PICF could be associated with the chronic inflammatory process of the peri-implant supporting tissues.

CONCLUSION.

Patients with peri-implantitis, apparently have an overregulation of the RANKL/BAFF-BLyS axis, which should be investigated in depth in further studies with a larger sample size.

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