Soluble RANKL in Crevicular Fluid of Dental Implants: A Pilot Study

Gabriel Monov, MD, DDS;* Georg D. Strbac, DDS;* Monika Baron, MD, DDS;* Barbara Kandler, MS;* Georg Watzek, MD, DDS;*† Reinhard Gruber, PhD*†

ABSTRACT

Background: Receptor activator of NF-κB ligand (RANKL), a member of the tumor necrosis factor superfamily, is a key mediator of osteoclast formation, activation, and survival. Thus, it is reasonable to hypothesize that there might be a functional relationship between RANKL expression and peri-implantitis.

Purpose: This pilot study was performed to determine the reference levels for soluble RANKL (sRANKL) in peri-implant crevicular fluid and to correlate them with the clinical parameters associated with inflammatory reactions and bone destruction.

Materials and Methods: The clinical parameters probing depth (PD), modified bleeding index (MBI), and modified plaque index (MPI) served as indicators for bone resorption and inflammation. Exclusion criteria for calculations were the detection limit of the immunoassay and the minimum acceptable crevicular volume for measurement. From the 84 collected samples of 16 patients, 30–84 years of age, with a total of 19 implants, 29 met these criteria. The absolute amount of sRANKL within crevicular fluid adsorbed to filter strips was a median of 0.18 femtomol (fmol; range, 0.08–0.53) and 0.26 nM (range, 0.09–1.21) when normalized by volume. PD was 4 mm in median and varied within a range between 2 and 12 mm.

Results: Absolute amounts of sRANKL showed no correlation with the adsorbed volume and the clinical parameters PD, MBI, and MPI. When sRANKL was normalized by volume, no correlation with the clinical parameters PD, MBI, and MPI was observed either. The patients' age was not associated with total sRANKL and the concentration of RANKL within crevicular fluid. Absolute levels of sRANKL and sRANKL concentration did not show any differences based on the sampling sites buccal and lingual, or on the patients' gender. A significant difference in sRANKL concentration was detectable when samples from maxillary implants (0.31 nM median; range, 0.12–1.21) were compared with samples from mandibular implants (0.21 nM median; range, 0.09–0.6) ($p = .03$). Absolute levels of sRANKL were not different between the maxilla and the mandible.

Conclusion: Given the limited sample size, our data provide a basis for future prospective longitudinal studies on the possible relevance of sRANKL as a prognostic marker in peri-implantitis, and for an understanding of the pathophysiologic process of the disease as a prerequisite for the design of treatment strategies.

KEY WORDS: inflammation, osseointegration, osteoclast, peri-implantitis, RANKL

*Department of Oral Surgery, Vienna Medical University, Vienna, Austria; †Ludwig Boltzmann Institute of Oral Implantology, Vienna, Austria

Reprint requests: Reinhard Gruber, PhD, Medical University of Vienna, Dental School GmbH, "Bernhard-Gottlieb Universitätszahnklinik," Department of Oral Surgery, Vienna, Austria, Währingerstraße 25a, AN-1090 Vienna, Austria; e-mail: reinhard.gruber@meduniwien.ac.at

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Osseointegration is a dynamic process that culminates in the formation of an intimate contact between the implant and the surrounding bone, and is a prerequisite for the clinical success of implant-supported prostheses.[1,2] A meta-analysis of prospective long-term studies indicates that the estimated survival of implants in implant-supported fixed partial dentures is 95.4% after 5 years, and 92.8% after 10 years.[3] Peri-implantitis and soft tissue complications occurred in 8.6% of the cases after 5 years.[3] Peri-implantitis is a

disease that leads to the progressive destruction of bone tissue and is the main cause of implant failures after functional loading.[3,4] Its etiology is related to persistent bacterial contaminations[5] that can cause chronic inflammation of the soft tissues surrounding the implant.[6,7] Prior to the manifestation of irreversible bone loss in peri-implantitis, peri-implant mucositis occurs, which refers to the reversible inflammatory reaction preceding peri-implantitis.[8] A reliable biochemical parameter to determine or predict the transition of reversible peri-implant mucositis to irreversible peri-implantitis would thus be of great clinical relevance.

Peri-implant crevicular fluid can be obtained under defined conditions by the insertion of filter strips into the space between the implant and the surrounding soft tissue.[9] Crevicular fluid provides a snapshot of the local biochemical peri-implant microenvironment that, together with the clinical parameters of chronic inflammation and bone resorption, can help diagnose and monitor disease progression.[4] Clinical parameters of peri-implant lesions can be characterized by pocket probing depth (PD), modified bleeding index (MBI), modified plaque index (MPI), and radiographic observations.[4] Inflammatory cytokines[10,11] and catabolic enzymes such as matrix metalloproteinases (MMP)-7, MMP-8,[12,13] MMP-9,[14] and elastase,[11] as they are released into the peri-implantitis crevicular fluid, can contribute to soft tissue destruction. However, bone resorption at the sites of chronic inflammation requires the presence and activity of osteoclasts.[15,16]

Osteoclast formation and activation is coordinated by the interaction of three members of the tumor necrosis factor (TNF) superfamily: the receptor activator of NF-κB ligand (RANKL), RANK, and osteoprotegerin (OPG).[17–19] Stromal cells, osteoblastic cells, and activated T-cells express RANKL in a membrane-bound form, which can be cleaved into a soluble form by the TNF-α-converting enzyme.[20] The membrane bound and soluble RANKL (sRANKL) can interact with their corresponding receptor RANK on progenitors of the monocyte-macrophage lineage. The activation of RANK signaling causes differentiation and fusion into mature osteoclasts and regulates the rate of resorption and survival. OPG is a soluble decoy receptor for RANKL and prevents the interaction with RANK. The cellular distribution of RANKL and OPG was described in diseases of chronic inflammation and progressive bone loss, such as rheumatoid arthritis,[21] periodontitis,[21] and loosening of orthopedic implants.[22–24] In periodontal disease, the RANKL protein was detected in leukocytes by immunocytochemical staining, mainly involving T-cells and macrophages that form large mononuclear cell infiltrates in the granulation tissue of periodontitis lesions.[25] OPG was associated with the cells that line the blood vessels. sRANKL and OPG were determined in crevicular fluid from patients with periodontal disease, which resulted in an increased sRANKL/OPG ratio.[26]

Until now, no data on the levels of sRANKL and OPG in peri-implant crevicular fluid have been available. We hypothesized that the ratio between sRANKL and OPG increases based on the clinical parameters of peri-implantitis. This pilot study was performed to determine the reference levels for sRANKL and OPG in peri-implant crevicular fluid and to correlate them with the clinical parameters associated with inflammatory reactions and bone destruction. This information provides insights into the underlying pathophysiologic mechanisms of peri-implantitis and illustrates whether sRANKL and OPG could serve as robust and sensitive clinical parameters for peri-implantitis.

MATERIALS AND METHODS

Study Population

Each of the 19 subjects between the ages of 30 to 84 years, with a total of 42 dental implants, were invited by the Department of Oral Surgery, Medical University Vienna, during an implant recall visit, were recruited for the study and asked to participate in one clinical examination. None of the patients had a history of systemic disease and had received antibiotics within the prior 4 months. All patients were nonsmokers. The medical and dental history was assessed using a structured questionnaire. Information was acquired regarding the occurrence of any systemic illness since implant placement, past and present medication, smoking status, pregnancy, and dental and periodontal treatment. Patients with overloaded prosthetic constructs were excluded from the study. A wide range of different prosthetic solutions with single implants, bridges, and implants connected by a gold alloy bar was mentioned. The protocol was approved by the ethical committee of the Medical University Vienna (EK#259/2004).

Collection of Peri-Implant Crevicular Fluid

Peri-implant crevicular fluid was collected with the intracrevicular method.[9] Implant surfaces were air-dried and isolated by cotton rolls. Paper strips (Periopaper, ProFlow, Inc., Amityville, NY, USA) were inserted into the crevice of implants for 30 seconds. The adsorbed volume was determined by impedance measurement (Periotron 8000, Oraflow, Inc., Plainview, NY, USA). Paper strips were placed into 1.5-mL plastic tubes containing 400 µL of phosphate-buffered saline and stored at −20 °C prior to analysis. After thawing, sRANKL was eluted by continuous shaking for 30 minutes.

Evaluation of the Peri-Implant Status by Clinical Parameters and Radiographs

All clinical parameters were collected and recorded by the same investigator (G.M.). The amount of plaque was scored using the MPI.[27,28] The following parameters were recorded at the buccal and the lingual site of each implant. Bleeding tendency of the marginal peri-implant tissue was evaluated using an MBI.[28] Peri-implant PD,[28] defined as the linear distance from the free mucosal margin to the bottom of the pocket, was recorded by a pressure-sensitive millimeter probe (Hawe ClickProbe®, tip diameter: 0.45 mm; probing force: 0.20–25 N; Hawe Neos Dental, Bioggio, Switzerland). In addition, radiographs were obtained from the implants based on the parallel, long-cone technique.

Determination of sRANKL and OPG in Crevicular Fluid

The immunoassay for sRANKL is based on a sandwich technique. OPG-coated microtiterplates capture sRANKL within the crevicular fluid, which is, in turn, detected by a labeled anti-RANKL antibody. The immunoassay is specific for sRANKL not previously bound to OPG[29] (Biomedica Gruppe, Vienna, Austria). The sensitivity of the assay for sRANKL is 0.08 pM. To calculate the original concentration of sRANKL in crevicular fluid, data were normalized by the adsorbed volume. The immunoassay for OPG (Biomedica Gruppe) has a detection limit of 0.14 pM.

Statistical Analysis

The Mann-Whitney test was used to compare sRANKL values in the two groups and the Spearman Rank Correlation test was used to find a correlation between sRANK concentration and the three clinical parameters. A paired *t*-test was used to compare sRANKL within the buccal and lingual sites of the same implant. Significance was assigned at the $p < .05$ level.

RESULTS

sRANKL in Peri-Implant Crevicular Fluid Does Not Correlate with the Corresponding Clinical Parameters

Total volume adsorbed to filter strips correlated with the clinical parameters PD ($R = 0.55$; $p < .01$), MBI ($R = 0.60$; $p < .01$), and MPI ($R = 0.49$; $p < .01$) (Figure 1A). No correlation between the clinical parameters and absolute amounts of sRANKL per filter strip was observed (PD [$R = 0.15$; $p = .45$], MBI [$R = 0.21$; $p = .28$], and MPI [$R = 0.07$; $p = .73$]) (Figure 1B). When sRANKL was normalized to the adsorbed volume, no correlation with the clinical parameters, PD ($R = –0.23$; $p = .22$), MBI ($R = –0.27$; $p = 0.16$), and MPI ($R = –0.36$; $p = .06$), was observed either (Figure 1C).

Median Values and Data Distribution

Of the 84 eluted crevicular fluid samples, 29 (35%) reached both criteria, the detection limit of the immunoassay for sRANKL of 0.08 pM, as well as a minimum adsorbed volume of 0.3 µL based on the impedance measurement. These 29 samples were obtained from 16 patients with a median age of 63.5 years (range, 30–84). Twenty-nine samples were obtained from the buccal and lingual site of 19 implants. Total amount of sRANKL per filter strip was 0.182 femtomol (fmol) (median; range, 0.08–0.53) and 0.26 nM (range, 0.09–1.21) when normalized by the adsorbed volume. The adsorbed volume was 0.83 µL (median; range, 0.3–1.14). No correlation between absolute sRANKL and the adsorbed volume was observed ($R = 0.06$; $p = .74$; Figure 2). OPG did not reach the detection limit of the immunoassay in either of the samples. PD was 4 mm (median) and varied within a range between 2 and 12 mm.

Correlation with Age, Comparison between the Buccal and Lingual Sites, Gender, and the Mandible and Maxilla

Patient age showed no correlation with either absolute amounts of adsorbed sRANKL ($R = –0.03$; $p = .88$) or sRANKL normalized to adsorbed volume ($R = –0.16$;

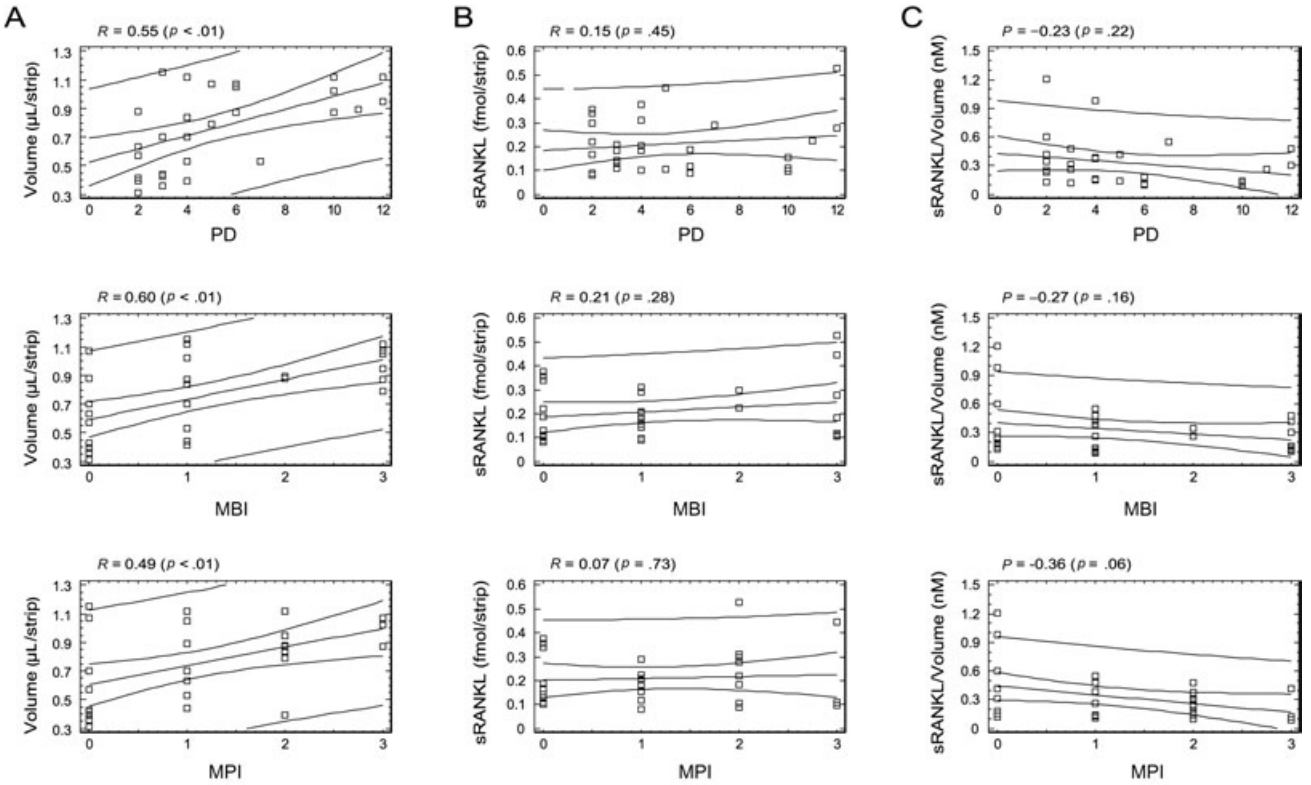

Figure 1 Relationship between (A) the volume of peri-implant crevicular fluid adsorbed to filter strips under defined conditions, (B) the amounts of soluble receptor activator of NF-κB ligand (sRANKL) adsorbed to the filter strips, (C) the concentration of sRANKL within the crevicular fluid, and the clinical parameters of peri-implantitis. The levels of sRANKL were determined by immunoassay. The volume on the filter strips was determined by impedance measurement. The regression lines and the 99 and 95% confidence intervals are shown. (PD = probing depth; MBI = modified bleeding index; MPI = modified plaque index)

p = .41) (Figure 3). Median absolute sRANKL of all samples included in the study was 0.17 fmol in the mandible and 0.21 fmol in the maxilla, with a range of 0.08–0.53 and 0.10–0.38, respectively (p = 0.38).

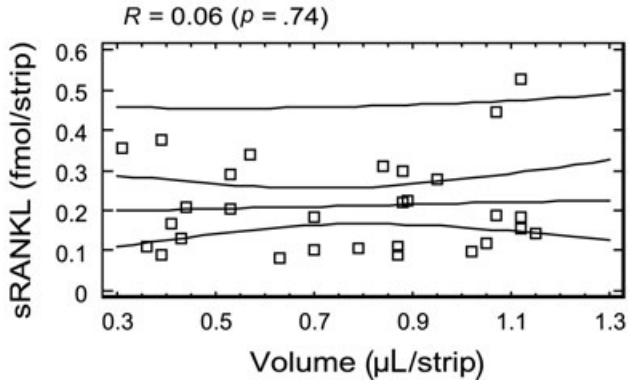

Figure 2 Relationship between the amount of soluble receptor activator of NF-κB ligand (sRANKL) adsorbed to the filter strips and the volume of peri-implant crevicular fluid adsorbed to filter strips under defined conditions. The levels of sRANKL were determined by immunoassay. The volume on the filter strips was determined by impedance measurement. The regression lines and the 99 and 95% confidence intervals are shown.

Median sRANKL normalized by the eluted volume was 0.21 nM in the mandible and 0.31 nM in the maxilla, with a range of 0.09–0.6 in the mandible and 0.12–1.21 in the maxilla (p = .03; Figure 4A). When absolute amounts of sRANKL were compared between lingual and buccal sites, median values of 0.22 fmol (range, 0.08–0.53) and 0.17 fmol (range, 0.09–0.34) were observed (p = .41). No differences between the sRANKL normalized by volume in the buccal (median, 0.31 nM; range, 0.09–1.21) and lingual (median, 0.25 nM; range, 0.12–0.6) sites were found (p = .84) (Figure 4B). Similarly, sRANKL levels did not differ within the buccal and lingual sites of the same implant (p = .08 for absolute sRANKL and p = .18 for sRANKL concentration; data not shown). Crevicular fluid obtained from male patients had a median absolute sRANKL of 0.24 fmol (range, 0.12–0.44), and in female patients, a median of 0.17 fmol (range, 0.08–0.53) (p = .10). sRANKL normalized by the volume had median values of 0.26 nM (range, 0.11–1.21) in males and 0.26 nM in females (range, 0.09–0.98) (p = .68) (Figure 4C).

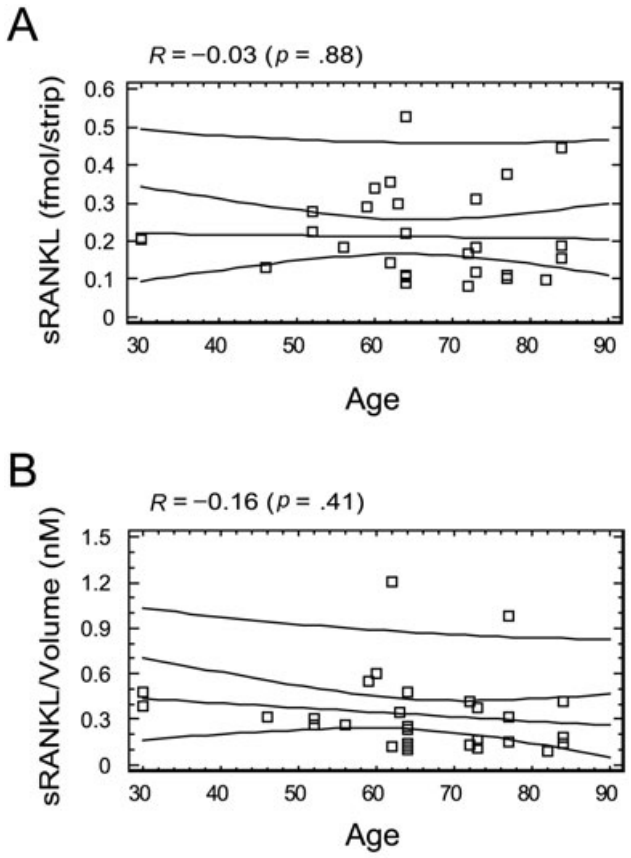

Figure 3 The relationship between (A) the amounts of soluble receptor activator of NF-κB ligand (sRANKL) adsorbed to the filter strips and (B) the concentration of sRANKL within the crevicular fluid with the age of the subject. The levels of sRANKL were determined by immunoassay. The volume on the filter strips was determined by impedance measurement. The regression lines and the 99 and 95% confidence intervals are shown.

DISCUSSION

Our pilot study was designed to assess the correlation of sRANKL in the peri-implant crevicular fluid with the clinical parameters of peri-implant bone loss and inflammation. RANKL is the key molecule of osteoclastogenesis in bone development, remodeling, and in pathologic conditions associated with chronic inflammation.[17–19] Although RANKL is expressed as a transmembrane member of the TNF superfamily, sRANKL can be released from the cell surface by proteolytic cleavage.[20] sRANKL, in turn, acts as a paracrine osteoclastogenic factor that can be detected by immunoassay.[26,30] We therefore hypothesized that the local inflammatory reaction and the bone loss of peri-implantitis are associated with increased levels of sRANKL within the crevicular fluid. However, no correlation between the total amount of sRANKL adsorbed to the filter strips or the concentration of sRANKL within the crevicular fluid with the clinical parameters of peri-implantitis was found in our pilot study. Characterization of the set of samples showed that sRANKL levels in samples obtained from the buccal and the lingual sites were not different, nor were there any differences based on gender. Only sRANKL concentrations, but not the absolute levels, were higher in samples obtained from the maxilla when compared with the mandible, suggesting possible differences between the anatomic regions. This might be important for future studies to avoid interfering influences due to sample inhomogeneity.

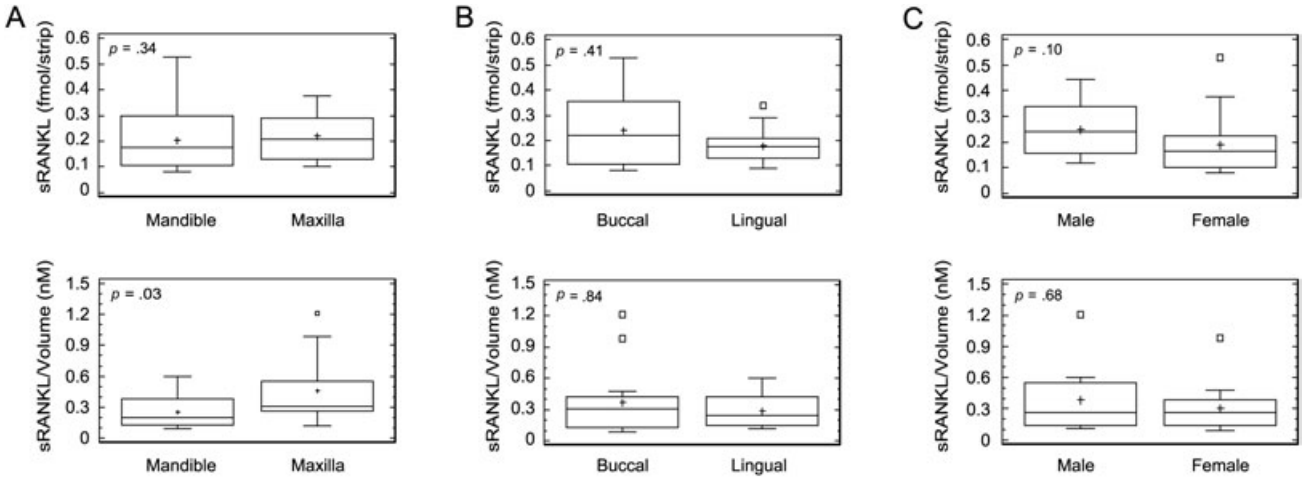

Figure 4 Concentration of the amount of soluble receptor activator of NF-κB ligand (sRANKL) adsorbed to the filter strips (upper graph) and the concentration of sRANKL within the crevicular fluid (lower graph) obtained from (A) the mandible and maxilla, (B) the buccal and lingual sites of the implant, and (C) from subjects of different genders. Data are presented as box-plots. The sRANKL levels were determined by immunoassay.

Our results show that the total adsorbed volume of crevicular fluid was higher at sites of detectable bone loss and inflammation, confirming findings that peri-mucositis and peri-implantitis are both associated with higher levels and flow rates of crevicular fluid.[9] In subjects with increased peri-implant bone loss and clinical signs of inflammation, however, the absolute levels of sRANKL and the concentration of RANKL within the crevicular fluid were not increased. These findings suggest that the immunoassay for sRANKL cannot successfully reflect the disease state. Overall, to identify subjects at increased risk for peri-implantitis and to allow a detailed subanalysis with high power, prospective longitudinal studies with a large sample size are necessary.

Nevertheless, an association between the expression of RANKL and the pathophysiology of peri-implantitis is reasonable to suggest. Inflammatory cytokines, such as interleukin (IL)-1 and TNF-α, which are highly abundant in crevicular fluid of peri-implantitis, are likely candidates to play a role in pathologic bone resorption.[11] Both IL-1 and TNF-α can potentiate the osteoclastogenic effect of permissive levels of RANKL under in vitro conditions.[31,32] T-cell-derived TNF-α was shown to be a central mediator of bone loss in osteoporosis models,[33] and the blocking of IL-1 and TNF-α are clinically approved in the treatment of rheumatoid arthritis, where chronic inflammation leads to joint destruction.[34] It is therefore possible that in peri-implantitis, inflammatory cytokines, together with sRANKL, account for the persistent bone loss that leads to implant failure. Inflammatory cytokines can also induce the expression of membrane-bound RANKL by osteoblasts, which is not released into the crevicular fluid and fails to be detected by immunoassay.[35] Further studies will focus on the question of whether increased levels of inflammatory cytokines potentiate the effects of sRANKL, and whether increased membrane-bound RANKL is a main inducer of osteoclastogenesis in peri-implantitis.

The use of impedance measurements allowed us to determine the amount of crevicular fluid volume isolated from peri-implant tissue. Due to the minimum acceptable fluid volume of 0.3 μL and the detection limit of the sRANKL immunoassay of 0.08 pM, 65% of the samples had to be excluded from further calculations. Hence, crevicular fluid in a very selected population of subjects was investigated; therefore, the results must be interpreted with regard to these limitations. However, the remaining samples from 16 subjects showed an equal distribution of adsorbed amounts of sRANKL and the clinical parameters, thus allowing a correlation analysis of this selected cohort. Further studies should take these limitations into account, indicating the need to pool at least two strips from one implant within a small elution volume. As OPG was not detectable in either of the samples, these recommendations are even more important. In view of the inherent limitations of this pilot study, the data can provide a basis for future studies with regard to the relevance of prognostic markers from crevicular fluid and for the understanding of the pathophysiologic process of the disease.

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