Histopathological Verification of Osteoimmunological Mediators in Peri-Implantitis and Correlation to Bone Loss and Implant Functional Period

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Peri-implantitis (PI) is characterized by inflammation and bone resorption eventually leading to implant failure, but the characteristic pathologic determinants are undefined to date. This study aims to elucidate the parameters involved in PI pathogenesis, including intraoral implant retention time, extent of bone loss, smoking history, and identification of osteoimmunological markers for inflammation and bone loss. Peri-implant tissues (n = 21) displaying clinically diagnosed PI from patients with vertical bone loss ranging from 0–12 mm and implant function period between 1 and 60 months were evaluated by histochemistry and immunohistochemistry for TRAP, CD3, RANK, RANKL, OPG, and TNF-α. Statistical analyses were performed with the Welch test and correlation coefficients were calculated. Most bone resorption occurred during the first 12 months of implant function and correlated with the extent of inflammation, although histological signs of inflammation strongly varied between samples from minimal appearance of inflammatory cells to extended infiltrates. Implant function period and smoking history did not significantly affect the degree of inflammation. Higher RANK levels emerged in the first 12 months of implant function compared to longer retention times and were negatively correlated to the occurrence of RANKL. Additionally, histological signs of inflammation were about two-fold higher in specimens with bone resorption up from 5 mm compared to under 5 mm. CD3⁺ cells were more prevalent in extensive inflammatory infiltrates and samples derived from smokers. Our analyses proved that PI-induced bone loss is differentially influenced by the parameters evaluated in this study, but a distinct interconnection between disease severity and implant retention time can be established.

Key Words: bone loss, inflammation, peri-implantitis

INTRODUCTION

Inflammation around dental implants is a biological complication of prosthodontic therapy during implant function time caused by bacterial infection. A plaque-induced mucositis restricted to the peri-implant soft tissues can lead to a peri-implantitis (PI), which is characterized by clinical signs of inflammation, such as bleeding on probing, pustulation, and progressive loss of supporting alveolar bone (which is the most important reason for late implant loss). PI pathogenesis resembles periodontitis: both are induced by a similar pathogenic microflora, are characterized by the release of proinflammatory mediators like prostaglandins or interleukins, and involve biomarkers associated with bone remodeling, such as tartrate-resistant acid phosphatase (TRAP). The histopathological appearances of these diseases are characterized by inflammatory infiltrates mainly consisting of T and B cells, plasma cells, polymorphnuclear neutrophile leucocytes (PMNL), and macrophages. However, only a few studies focus on molecular markers potentially involved in PI pathogenesis.

In the field of osteoimmunology, which investigates the impact of immunological factors on bone metabolism and osseous architecture, the RANK/RANKL system has emerged as a central dogma interacting with many other anabolic or catabolic factors for the regulation of bone remodeling in health and disease. The receptor activator of nuclear factor kappa-B ligand (RANKL) is localized on different cells, including osteoblasts or lymphocytes, and interacts with its physiological RANK receptor, located in the cell membrane of osteoclasts and their mononuclear precursors. Binding of these 2 elements induces osteoclast differentiation and activation of mature osteoclasts. RANKL can be regulated by osteoprotegerin (OPG), which is secreted by osteoblasts as a decoy receptor bind and sequester RANKL, thereby limiting the biological activity of the latter. Components of the RANK/RANKL system have also been
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**TABLE 1**

Clinical data of the specimens with clinically diagnosed peri-implantitis

<table>
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<tr>
<th>Specimen Number</th>
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detected in healthy and diseased orofacial tissues, where they play a role in tooth eruption and are involved in the remodeling of alveolar bone and the periodontal ligament (PDL).16–18 As the RANK/RANKL system plays a central role in the mediation of bone loss in periodontitis and as soluble RANKL was found in the crevicular fluid of patients with PI,19,20 the RANK/RANKL system is likely to hold a major function in PI pathogenesis. However, a histopathological profile of inflammatory and osteoimmunological markers in PI has not yet been characterized.

Additionally, several studies highlighted the significant impact of smoking history on PI pathogenesis with increased susceptibility for peri-implant marginal bone resorption and late implant failure, whereas other investigations could not observe any correlation between smoking and PI severity.21–23

The aim of this study was to elucidate the parameters involved in PI pathogenesis via histology and immunohistochemistry in tissues from patients with clinically diagnosed PI. In these samples, special regard was given to intraoral retention period of the implants, extent of bone loss, smoking history, and occurrence of osteoimmunological markers for bone loss and inflammation. In addition, potential correlations were investigated to clarify the specificity of PI for a more definite understanding of its onset and progression.

**Material and Methods**

**Patients and collective material**

The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki, with the understanding and written consent of each subject. The study has been independently reviewed and approved by the ethic committee of the University of Bonn.

Peri-implant tissues of systemically healthy patients (n = 21; mean age, 58 years) with clinically diagnosed PI by the parameters bleeding on probing, probing depth > 4 mm and loss of attachment level > 3 mm were collected.24,25 The distance from the implant platform to the first visible bone-implant contact was measured mesially and distally, at which the implant length was the reference.

Six of the patients were smokers and none had used antibiotics within the previous 6 months. The implants had been in function between 1 and 60 months. Vertical bone loss ranged from 0 to 12 mm and was determined via panoramic radiographs, as they were necessary for patients’ basic check-up of anatomical structures (including teeth, implants, maxillary sinuses, and nasal floor), as well as for determination of general occurrence of periodontitis or bone resorption.26

The peri-implant tissue specimens were obtained with established external gingivectomy surgical procedures. Control samples comprised clinically healthy gingiva derived from tooth extractions and clinically healthy peri-implant gingiva from 2 patients each. Corresponding clinical data are listed in Table 1.

**Histology, TRAP histochemistry, and immunohistochemistry**

Following fixation and dehydration in an ascending series of ethanol, specimens were paraffin-embedded and serially cut in sagittal sections of 5 μm. Selected sections were stained with haematoxylin-eosin (HE). In order to identify osteoclast-like cells, selected tissue sections were stained for tartrate-resistant acid phosphatase (TRAP) activity, according to Barka and Anderson.27

Furthermore, selected slides were processed for the immunohistochemical detection of CD3, RANK, RANKL, OPG, and TNF-α, after digestion with 0.4% pepsin. Incubation with the antihuman primary antibodies was performed at previously optimized concentrations. Chromogen staining of bound antibodies was done using the peroxidase-conjugated EnVision antimouse system (Dako, Glostrup, Denmark) or EnVision antirabbit and antigoat horseradish peroxidase-conjugated secondary antibodies (Dako). Peroxidase activity was visualized using 3,3′-diaminobenzidine (DAB), yielding a brown staining product before sections were counterstained with Mayer’s haematoxylin.

Negative control samples were subjected to each staining procedure by treating another tissue section of the same slide with TBS instead of the primary and secondary antibody, respectively.

Images were captured by transmitted-light microscopy (Axioskop 2, Carl Zeiss Jena GmbH, Jena, Germany).

**Histomorphometry**

Two independent investigators carried out a blinded histological evaluation of each biopsy in 3 different high power field subepithelial regions of interest at a magnification of ×40. In HE-stained sections, inflammation was graded into 5 stages: 1 = normal, 2 = minimally increased number of inflammatory cells, 3 = moderately increased number of inflammatory cells...
and distinct infiltration aggregates, 4 = markedly diffuse infiltrates, 5 = extended infiltrates. RANKL and TNF-α immunostaining intensity was classified semiquantitatively (0 = no staining, (1) = moderate staining, + = strong staining, ++ = very strong staining). The number of cells with positive staining reactions or immunoreactions was counted for the markers TRAP, CD3, RANK, and OPG.

Three images per specimen were analyzed and the mean was calculated for each sample.

**Statistical analysis**

For statistical analyses, the specimens were divided into 3 groups corresponding to the intraoral retention period of the implants, defined as “group A” for implants up to 12 months in situ, “group B” between 13 and 24 months, and “group C” over 24 months in situ. For evaluation of the role of inflammation, samples were divided in “group a” for gradings comprising “none” and “moderate,” and “group b” for gradings comprising “strong” and “very strong” inflammation patterns. Investigations on bone loss were performed by building “group 1” for specimens with bone loss up to 5 mm, and “group 2” with bone loss equal to or over 5 mm. Furthermore, comparisons were made between smokers (group s) and nonsmokers (group ns).

Statistical evaluation was performed using a Welch test for detection of statistically significant differences between paired observations. Values were calculated for n = 21 and are expressed as mean ± SD. P < 0.05 was considered statistically significant.

Correlation coefficients were calculated to evaluate the statistical relationship between the different variables investigated. Analytic tests were performed using SPSS (version 19).

**RESULTS**

**Histology**

Specimens of clinically healthy gingiva displayed normal structures characterized by a stratified squamous epithelium supported by a vascular lamina propria, which is representatively shown in Figure 1a. Specimens of clinically healthy peri-implant gingiva (Figure 1b) comprised fragments of keratinized oral epithelium with moderate rete ridges and a vascular dense connective tissue lamina propria with moderate mixed cell infiltrates. Bony fragments and small ectopic calcifications of different sizes were scattered within the connective tissue.

Samples of PI tissue consistently manifested subepithelial infiltrates dominated by plasma cells (Figure 1c). Infiltrations were only moderate in most cases, and samples with extensive inflammatory infiltrates were very dense and intermingled with the rete ridges or penetrated between the epithelium. Due to the surgical techniques, an intact structural topography could not be maintained in all PI samples; however most of them possessed a continuity of a wide keratinized oral epithelium with rete ridges and a thin pocket epithelium that was partly ulcerated or discontinuous in many cases. The samples exhibited vessels of thin and distended venules, but also partly presented with thickened walls and perivascular hyalinization. Frequently, bone fragments could be found at the periphery of the specimens opposite to the epithelium, indicating an apical-crestal direction.

**TRAP histochemistry**

The TRAP positive cell population consisted of mononuclear infiltrate cells with characteristics of macrophages and a few multinuclear osteoclasts located in resorption lacunae of bone fragments or ectopic calcifications (Figure 2).

**CD3**

In clinically healthy gingival and peri-implant tissues, very few to no scattered subepithelial positive lymphocytes could be identified (Figure 3a). In most PI specimens, dense or loosely packed clusters of CD3⁺ cells appeared in inflammatory infiltrates as much as in subepithelial areas, which is representatively shown in Figure 3b. However, a few specimens only exhibited low numbers of CD3⁺ cells.

**OPG**

OPG was stained weakly to moderately in cells of the epithelial layer in clinically healthy gingival and peri-implant tissues (Figure 3c). In PI, epithelial immunostaining was more intense.
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**FIGURE 2.** Histochemical photomicrograph of tartrate-resistant acid phosphatase (TRAP) stained gingival structures. TRAP positive cell populations comprising macrophages (arrows) and cells with osteoclast-like morphology (arrowheads) scattered in gingival structures. Original magnification ×20.

and additionally, OPG immunoreactivity was observed in subepithelial fibroblasts, vessel walls and cells of inflammatory infiltrations (Figure 3d).

**RANK**

In clinically healthy gingival and peri-implant tissues, moderate RANK immunoreactivity was evident in the suprabasal layers of the stratified keratinized epithelium, in the subepithelial extracellular matrix, and in vessel walls. An exemplary photomicrograph is shown in Figure 3e. In PI, a similar immunostaining pattern was observed; however, extracellular immunoreactivity occurred in the subepithelial lamina propria and in bony fragments (Figure 3f).

**RANKL**

Weak to moderate immunostaining could be focally observed in the cells of the suprabasal layers of the epithelium, in few subepithelial fibroblasts, and in vessel walls of samples from normal gingival and peri-implant tissues (Figure 3g). In PI, RANKL was also present in epithelia and in some subepithelial fibroblasts. Besides, mononuclear cells in infiltrates showed moderate to strong immunostaining. A representative picture is provided in Figure 3h.

**FIGURE 3.** Immunohistochemical photomicrographs of healthy and diseased gingival structures. (a) CD3 staining in healthy gingiva comprising epithelium (E) and gingival connective tissue (G) revealed only few positive lymphocytes (arrowheads). Original magnification ×40. (b) CD3 positive cells (arrowheads) in extensive immune cells infiltrations of PI tissues. Original magnification ×20. (c) Moderate OPG verification in epithelial structures (E) of healthy gingiva and absence in gingival connective tissue (G). Original magnification ×40. (d) Intense OPG immunostaining in cells of inflammatory infiltrates (I) and in the extracellular matrix (E) in peri-implantitis (PI) tissues. Original magnification ×40. (e) RANK immunoreactivity in the subepithelial gingival connective tissue (G) and weakly in epithelial structures (E) of clinically healthy peri-implant tissues. Original magnification ×40. (f) Considerable confirmation of RANK in the subepithelial gingival connective tissue (G) and in bony fragments of a specimen with PI diagnosis. Original magnification ×20. (g) Focal RANKL verification in the epithelial layer (E) and absence

Specimens derived from clinically healthy gingival and peri-implant tissues featured weak to moderate TNF-α staining focally in epithelial cells, fibroblasts, and vessel walls of the subepithelial layers, as well as extracellularly in the subepithelial connective tissue matrix (Figure 3i). This staining pattern was similar in PI specimens, where the intensity was stronger and comprised of mononuclear cells in infiltrates with moderate to weak immunostaining (Figure 3j).

**Correlation of bone loss, inflammation, and influence of smoking**

Our analyses proved that PI-induced bone loss is differentially influenced by the parameters evaluated in this study. Investigations on the intraoral retention time of the implants revealed that most bone resorptive activity seems to occur in the first months of implant function, which is illustrated in Figure 4a. A statistically significant difference in the amount of bone loss was evident between “group A” with 5.0 ± 3.7 mm and “group B” with 10.0 ± 0.0 mm, whereas no significant variation could be detected between “group B” and “group C” with a mean bone loss of 7.8 ± 2.6 mm.

With regard to the extent of inflammation, a strong interdependence between the amount of bone loss and the dimension of the inflammatory infiltrate could be demonstrated. Results showed that “group a” displayed a significantly lower bone loss with 3.6 ± 3.4 mm compared to “group b” with a mean resorption of 8.0 ± 2.7 mm. Generally, the extent of inflammation varied between the samples: 7 specimens exhibited only minimally increased numbers of inflammatory cells, 5 biopsies showed a moderately increased number of inflammatory cells and distinct infiltration aggregates, 1 sample displayed markedly diffuse infiltrates, and 8 specimens presented even extended infiltrates. Additionally, the amount of bone loss significantly correlated with the histological signs of inflammation, as they were about 2-fold higher in specimens with bone resorption of 5 mm or more, compared to those with less than 5 mm. On the contrary, implant function period and smoking history did not significantly affect the degree of inflammation. Correlating smokers and nonsmokers, no statistically significant differences in the amount of bone deficit could be noted, revealing a mean bone loss of 5.8 ± 3.4 mm for “group s” versus 6.8 ± 3.7 mm for “group ns.”

**Correlation of immunohistochemical findings**

A closer investigation of the inflammatory and immunological markers involved in PI pathogenesis indicated significantly
in gingival connective tissue (G) of healthy gingiva. Original magnification ×20. (h) Distinct RANKL staining in the epithelium (E) and moderate verification in inflammatory cell infiltrates (I) of the subepithelial gingival connective tissue (G) affected by PI. Original magnification ×20. (i) Focal cellular and extracellular TNF-α staining in subepithelial gingival connective tissue (G) of peri-implant healthy gingiva. Original magnification ×20. (j) Strong TNF-α staining in the epithelium (E) and in inflammatory infiltrates (I) of PI tissues. Original magnification ×20.
higher levels of RANK in specimens derived from implants up to 12 months in function, as compared to longer retention time. Furthermore, significantly higher numbers of CD3\(^+\) cells were observed in samples with inflammation patterns graded as “strong” to “very strong,” which were on average 2.7-fold higher compared to moderate inflammation patterns. Similarly, CD3 immunoreactivity was 2-fold higher in samples derived from smokers when compared to nonsmokers. Calculations of the correlation coefficients demonstrated once more that the incidence of CD3 positive cells positively correlated with the extent of inflammation. Additionally, evaluations showed a negative correlation between the occurrence of RANK and RANKL. The results of the correlation coefficient calculation are listed in Table 2.

**DISCUSSION**

The present study investigated the parameters involved in PI pathogenesis, with regards to intraoral retention period of the implants, extent of bone loss, smoking history and occurrence of osteoimmunological and inflammatory markers. Additionally, potential correlations of these factors were evaluated. To date, the pathophysiology of PI is largely unknown, since valid data on clinical treatment strategies of peri-implant inflammatory disease do not exist to date.\(^{28}\)

Our analyses revealed that most bone resorption occurs during the first 12 months of implant function and can be correlated to the extent of inflammation. Data from studies on the mean implant function time range from 5 to 20 years, and correlate a PI disease prevalence of 5% to 43.3%.\(^{1}\) Our histological analyses revealed that signs of inflammation strongly varied between the samples investigated, ranging from minimal appearance of inflammatory cells to verification of pronounced inflammatory infiltrates. Histological signs of inflammation were about 2-fold higher in specimens with bone resorption greater than or equal to 5 mm, compared to those less than 5 mm. Implant function period and smoking history did not significantly affect the degree of inflammation.

Investigations comparing periodontitis and PI pathogenesis showed some similarities but also major differences in their disease patterns, as similar amounts of plaque cause more severe disease progression in PI.\(^{29}\) Furthermore, some periodontal pathogens can be found more frequently in PI, namely *Staphylococcus spp, Enterobacter, Klebsiella, and Candida spp*.\(^{1}\) Furthermore, the inflammatory infiltrate establishing a chronic disease can spread directly into bony structures in PI due to direct bone-to-implant contact, and lack of a protecting structure like the periodontal ligament more severely affects bone metabolism.\(^{30,31}\)

To date, no consistent inflammatory markers have been identified for PI. However, our results evidenced higher levels of RANK in an early stage of implant function compared to longer retention times, which had a negative correlation concerning the occurrence of RANK and RANKL. These findings agree with other investigations revealing that PI features significantly higher RANK levels, whereas periodontitis manifests significantly higher sRANKL concentrations and sRANKL/OPG ratios.\(^{31}\) These facts might be attributed to the increase of inflammatory mediators in PI that provoke activation of the RANK receptor, which in turn stimulates the biosynthesis of additional pro-inflammatory molecules and presumably results in a final stimulation of osteoclastogenesis.\(^{31,32}\) Consequently, the high levels of RANK particularly seen in PI might represent a characteristic feature for the regulatory processes in bone resorption seen in this inflammatory disease.\(^{31,33}\) Analyses on RANK concentrations in the crevicular fluid of periodontally healthy versus inflammatory diseased individuals showed that the average RANK levels were significantly higher in PI and even higher compared to patients with periodontitis.\(^{34}\)

Immunohistochemical analyses of our study also manifested increased staining intensities for TNF-\(\alpha\) in PI specimens compared to samples of healthy gingiva. TNF-\(\alpha\) is assumed to be important for the osteolytic processes of PI and shows increased prevalence in patients with implant failure due to PI compared to controls.\(^{35}\)

The results of our study additionally revealed significantly higher ratios of CD3 positive cells in specimens with extensive inflammatory peri-implant infiltrates and in specimens derived from smokers. This opposes the theory that smokers exhibit an depressed immune system and are more susceptible to diseases like osteoporosis related to reduced numbers of bone marrow lymphocytes.\(^{36}\) However, evidence shows that smoke exposure decreases the number of B cells and enhances the ratio of CD8\(^-\) T cells compared to CD4\(^+\) T cells, but does not exert any effect on other lymphocyte subsets.\(^{36}\) The immunological hyper-responsiveness of smokers seen in our study might represent a protective autoregulatory feedback loop in the mechanisms involved in PI disease progression.

Some aspects of the present study merit consideration, such as the clinical limitations of an in vivo investigation with an inhomogeneous patient pool in some respects. Although all PI treatment cases of the office emerging during the recruitment time of the study were included, a total of 21 patients still represent a limited sample size for drawing general conclusions from the study. Nevertheless, our results give an initial idea of the osteoimmunological processes and mediators involved in peri-implantitis that need to be clarified in further research. A second limiting aspect is the nonstandardized implant placement procedure, including varying screw positioning and
angulation and implant length and diameter, related to differences between patient conditions and treatment needs.

**CONCLUSION**

Our results indicate that peri-implant bone loss exhibits a disease-specific pattern, and that PI pathogenesis apparently manifests characteristics distinct from other inflammatory diseases of the periodontium. PI-induced bone loss is seen to be differentially influenced by the parameters investigated in this study, but a distinct interconnection between disease severity and implant retention time can be established.

Thus, our study contributes to a better understanding of the host response in peri-implant tissue inflammation, and elucidates the characteristic features of PI concerning its bone degradation pattern and the modulating factors involved in disease severity and progression.

**ABBREVIATIONS**

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<th>Definition</th>
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<tr>
<td>TRAP</td>
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<td>OPG</td>
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**REFERENCES**

24. Konemann A, Beyer M, Deschner J, et al. Human periodontal ligament cells facilitate leukocyte recruitment and are influenced in their


