Tenascin-C and Matrix Metalloproteinase-9 Levels in Crevicular Fluid of Teeth and Implants

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ABSTRACT

Background: The role of and interaction between bacterial infection and biomechanical impact in the development of peri-implant inflammatory processes is not clear.

Objective: To determine the amount and concentration of tenascin-C (TNC) in gingival crevicular fluid (GCF) around teeth and in peri-implant sulcus fluid from healthy implants and implants with peri-implantitis, and to correlate it with matrix metalloproteinase-9 (MMP-9) levels.

Materials and Methods: Seven control individuals and 18 patients with 41 implants with/without peri-implantitis were included. GCF was collected with filter strips and volumes were measured with a Periotron device. The amount of serum albumin per sample was quantified by densitometric analysis of Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Relative activity of MMP-9 was determined from the densitometry of zymograms. Amounts and concentrations of TNC were evaluated by ELISA.

Results: Relative MMP-9 activity was increased in peri-implantitis. A tendency was observed to measure higher TNC concentrations at teeth than at implants. The amount of TNC in GCF collected from healthy implant sites and the peri-implantitis sites was significantly different. Based on immunoblotting, TNC in GCF seemed degraded. In contrast to TNC, MMP-9 was significantly related to the PD and the volume of GCF.

Conclusion: TNC is known to be induced in inflammation. The increase found in peri-implantitis was less than expected. In the context of peri-implantitis, TNC might be a marker of bone remodelling rather than inflammation and infection. A possible proteolytic degradation of TNC during peri-implantitis needs to be studied.

KEY WORDS: dental implants, gingival crevicular fluid, MMP-9, tenascin-C

INTRODUCTION

Traditional periodontal clinical parameters are used to describe peri-implantitis. Clinical measurements like bleeding on probing provide information regarding the presence of attachment and bone loss. However, these techniques do not identify sites actively undergoing disease progression or those sites at risk of deterioration. Host factors in the peri-implant sulcus fluid (PISF) associated with anatomical events of peri-implantitis might be useful for identifying and predicting future disease progression.

Otherwise, in a small fraction of patients with dental implants, chronic (aseptic) inflammation can lead to bone loss around the implant that results in its loosening of the implant and eventual failure.[1] In most cases, the etiology of such aseptic implant loss is not known but a biomechanical impact is suggested. Typical clinical signs of peri-implantitis are like increased probing depths, active suppuration, redness and swelling of the soft tissue, and bleeding on probing are not visible. One study done showed that the connective tissue around failed implants, which lacks signs of peri-implant infection, is surrounded by macrophages and

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cells participating in an immune response, and that epithelium often proliferates apically leading to encapsulation of the implant. The role of microorganisms in these processes remains unclear.[2] Thus, there are two phenotypes of bone loss.

The search for reliable methods of early detection of dental implant failure to enable quick intervention is ongoing. Clinical parameters, for example, pocket probing depth (PD), attachment level (AL) measurements, and bleeding on probing (BoP), have been used for the diagnosis and monitoring of disease progression.[3,4] However, clinical parameters and radiographs provide limited information about the dynamic pathophysiological mechanisms of disease initiation and progression. Therefore, simple and reliable clinical tests are needed to monitor peri-implant tissue health during recall visits.[2] There is a need for detecting molecular markers in the PISF that might indicate increased tissue turnover. Enzymes involved in extracellular matrix (ECM) turnover, as well as ECM components (or their proteolytic fragments) themselves, are obvious candidate molecules that could serve as diagnostic tools indicating aseptic implant loosening.

The turnover of bone extracellular matrix is controlled by a family of matrix metalloproteinases (MMPs) that consists of collagenases, gelatinases, stromelysins, and membrane-type MMPs.[5] During bone development and remodeling, MMP activity is required for matrix resorption and coupling to bone formation.[5–7] MMP-9 (gelatinase B) has been reported to affect the differentiation of osteoclasts and their recruitment into remodeling bone.[7–11] Because of their role in bone resorption, both MMP-9 and MMP-2 (gelatinase A) have been implicated in the loosening of prostheses.[12]

TSC is a large multifunctional ECM protein with a restricted distribution during development and disease.[13] It is specifically associated with early tooth development and later accumulates in periodontal ligament and periosteal tissue.[14–17] The expression of tenascin-C (TNC) is known to be induced on the one hand by inflammatory mediators such as interleukins, lysophosphatidic acid, and thrombin, and on the other hand, by mechanical stress in bone, ligaments, and tendons.[18] It is a reasonable assumption that both abnormal mechanical load and inflammatory reactions affect the expression of TNC in peri-implant tissue.

The aim of the study was to test the hypothesis that the sulcus exudate of affected implants reveals increased levels not only of MMP-9 but also of TNC.

MATERIALS AND METHODS

Study Subjects

Regular recall patients at the Department of Prosthodontics, School of Dental Medicine, Bern were recruited for the study. They all had natural teeth, and received at least one implant (Straumann Dental Implant System) to support a prosthesis. The selection was performed by computer-randomized numbers, which were based on their patients' chart numbers. Eighteen patients could be recruited with a total of 41 implants, which had successfully supported their prosthesis for at least 24 months. Exclusion criteria were: any systemic conditions that placed an individual in a high-risk category or would affect the inflammatory marker measurements (pregnancy, diabetes, hypertension, any inflammatory diseases), and use of antibiotics or anti-inflammatory medication within the preceding 3 months. The patients had to be healthy and had to confirm by signature that they were non-smokers or had stopped smoking before the implant therapy was started. After completion of the treatment, they had regularly participated at the maintenance program throughout the observation period at the department and were not lost from recall. This was confirmed by the records in the patients' charts. Based on the selection criteria, a total of 18 patients were eligible for the study and selected. They all gave their informed consent to participate in the study. The median age of the patients at the time of the study was 72.9 ± 10.9 years. When the patients were examined in the context of this study, they had neither received professional hygiene procedures by the dental hygienist within the last 4–6 months before data collection for this study, nor any specific therapy due to acute manifestation of periodontitis/peri-implantitis. A control group of seven healthy staff members from the dental school with natural teeth was selected and 14 teeth were measured in these probands.

Periodontal and Peri-Implant Parameters

The clinical parameters including PD, attachment loss (AL), and dichotomous evaluation of BoP were measured after the sampling procedure.

They were recorded again at mesiobuccal and distobuccal sites of teeth and implants in each subject. PD was measured from gingival/peri-implant mucosal margin to the bottom of the pocket with a calibrated probe. BoP was recorded (+), if present and (–), if absent. AL is the sum of PD and the positive or negative distance from the free marginal of gingiva/mucosa to a defined landmark on teeth or implants. Radiographs were also taken, but crestal bone measurements were not the parameter of interest in this study.

Shallow pockets and absence of BoP are used as parameters that indicate periodontal/peri-implant stability. Increased PD and particularly BoP, or a combination of both, are considered to be indicators for an inflammatory process with infectious disease. Therefore, for further comparison, a classification into three categories of the implant sites according to the periodontal parameters BoP and PD was established:

In the test groups with a total of 41 implants, 82 sites were measured. These sites were classified as described below:

- Classification l: implant sites with PD ≤ 3 mm and BoP (–), indicating healthy conditions of implants;
- Classification II: mixed findings; implants sites with either PD ≤ 4 mm and BoP(+)or PD ≥ 4 mm and BoP (–), indicating risk of peri-implantitis;
- Classification III: implant sites PD ≥ 5 mm and BoP(+); according to standard parameters in periodontology, classification III means overt signs of peri-implantitis.
- Control group: only tooth sites with PD ≤ 3 mm and BoP (–) were included, indicating healthy conditions of teeth.

Gingival Crevicular Fluid (GCF) and PISF

In the present study, known protocols for collecting crevicular fluid were adopted.[19] GCF and PISF were collected with sterile paper strips (Periopaper® strips, Pro Flow, Amityville, NY, USA). Periopaper® strip was inserted into the crevice and left in situ for 30 seconds and then transferred for volume determination to the chair-side located Periotron® 6000 (Harco Electronics, Winnipeg, Canada), which had been calibrated with known volumes of buffered solution.

Samples were obtained at mesiobuccal and distobuccal sites from all selected teeth and implants. Prior to the sampling, the area was gently dried with an air syringe and isolated by cotton rolls. If any obvious plaque was present on the visible surface of the implants and teeth, it was carefully removed with a curette. The volume was expressed in microliter (μL). Strips contaminated with blood were discarded. After collection, the strips were immediately placed into sterile Eppendorf tubes containing ice-cold 250 μL of buffered saline consisting of 0.01 M Na H_2PO_4 and 150 mM NaCl, pH 7.4. The fluid was eluted from the paper strips by vortexing the samples for 30 seconds, and centrifuged at 800 g for 10 minutes. The supernatant was removed from the vials, and supernatant kept at −80°C until analysis.

Densitometric Analysis of Serum Albumin in Crevicular Fluid Samples

Human serum albumin (HSA) is the major protein species in crevicular exsudate.[20,21] It is readily detected as a 68 kDa band when running samples on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Figure 1). To determine the amount of serum albumin in each individual sample, 30 μL aliquots of the protein eluted from the paper strips were run on 7.5% acrylamide-SDS mini slab gels under reducing conditions. Gels were stained for 30 minutes with 0.1% Coomassie Brillant Blue R (Serva, Heidelberg, Germany) and de-stained for 1 hour, both in 10% isopropanol 10% acetic acid. Gels were scanned with an Epson scanner, and the integrated density of the major 68 kDa band in each sample was determined using Image Quant software (Perkin Elmer, Waltham, MA, USA). An internal standard (10 μg) bovine serum albumin (BSA; Merck, Darmstadt, Germany) was included on each gel. The amount of serum albumin per sample was calculated from standard curves. These were produced by running different amounts of a serum albumin stock solution (crystalline BSA, 1 mg/mL in phosphate buffered saline [PBS], 0.68 OD_{280}; Fluka, Buchs, Switzerland) on SDS-PAGE gels which were processed and analyzed identically to the sample gels.

Gelatin Zymography for Determining the Relative Activity of MMP-9

Thirty microliter aliquots of crevicular fluid proteins eluted from individual paper strips were run on 7.5% polyacrylamide-SDS gels containing 0.1% swine skin gelatin (Merck) under non-reducing conditions. Gels

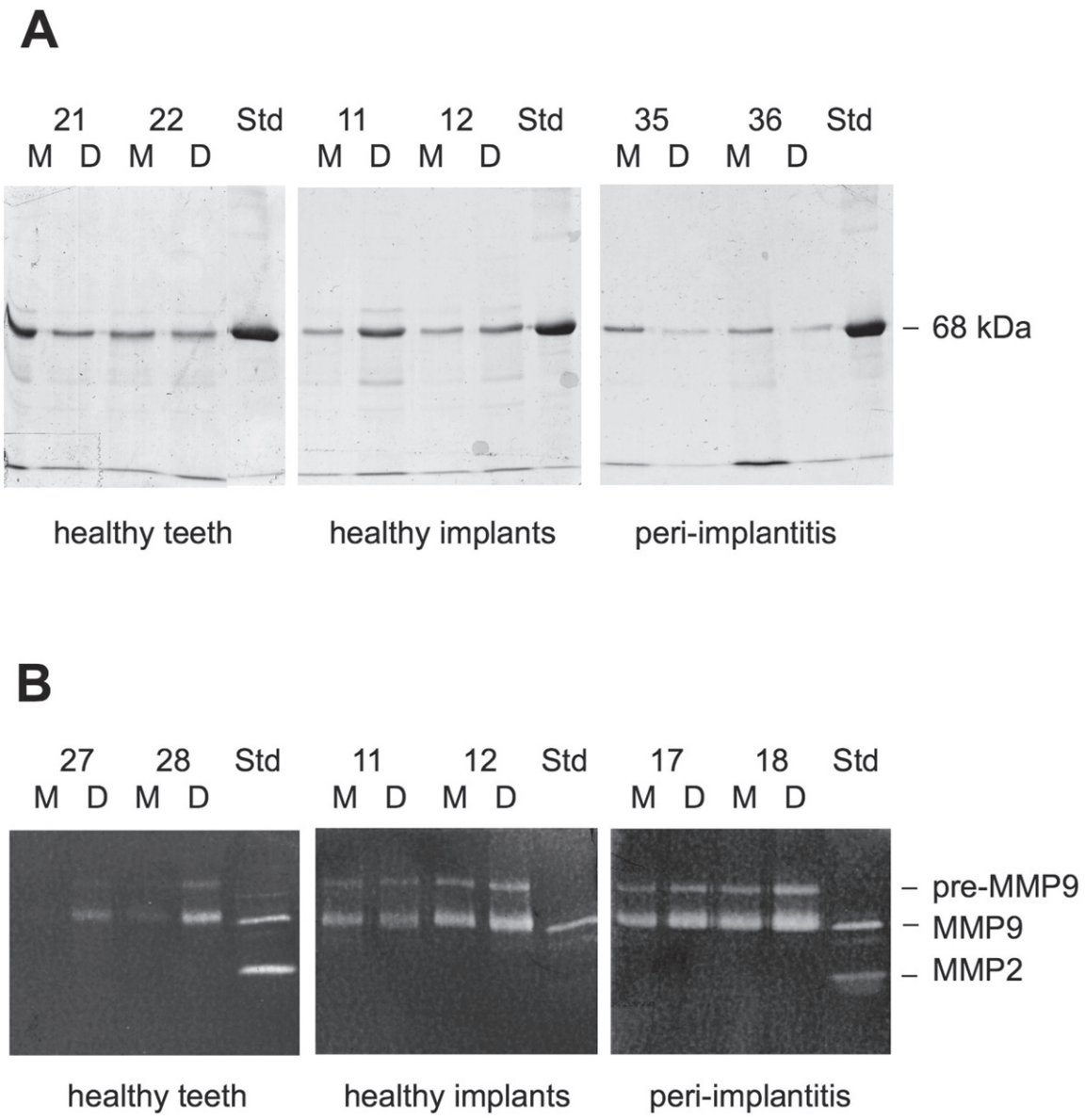

Figure 1 Determination of serum albumin content (A) and metalloproteinase (MMP)-9 activity (B) in gingival crevicular and peri-implant sulcus fluid. In both (A) and (B), results from randomly chosen patient samples are shown to exemplify the quantification methods. (A) Gingival crevicular fluid from individuals with healthy teeth, and peri-implant sulcus fluid from patients with healthy implants or with peri-implantitis, respectively, were collected as described in Materials and Methods. Aliquots were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels that were stained with Coomassie. The major human serum albumin band (68 kDa) was scanned for each sample, and the amount of protein quantified from comparison with bovine serum albumin standards (Std; 10 μg) run on the same gel. (B) Control, healthy implant and peri-implantitis samples (see above) were run on SDS-polyacrylamide gels, and gelatin zymograms were prepared as described in Materials and Methods. The positions of pre-MMP-9, MMP-9, and MMP-2 activities in the zymograms are indicated. On each gel, a MMP-9/MMP-2 standard (Std) obtained from fibroblasts conditioned medium was run for control. Relative MMP-9 activity was quantified from scans of the respective band in each patient sample. Both in (A) and (B), numbers on top of the gels indicate teeth/implants of individual patients; samples from mesiobuccal sites are labeled with M, those from distobuccal sites with D.

were washed twice for 30 min in 2.5% Triton X-100, 10 mM Tris-HCl, pH 8.0, and twice for 5 min in 50 mM Tris-HCl, pH 8.0. Gels were then incubated in metalloproteinase enzyme buffer (150 mM NaCl, 5 mM CaCl2, 50 mM Tris-HCl, pH 8.0) for 24 hours. Gels were developed by staining with 0.1% Coomassie and destaining, and the integrated density of the 92 kDa MMP-9 bands were determined as described above. 30 μL of a single batch of chick embryo fibroblast conditioned medium revealing pre-MMP-9 (120 kDa), MMP-9 (92 kDa), pre-

MMP-2 (72 kDa) and MMP-2 (66 kDa) bands was used as internal standard on each gel.[22]

ELISA for Quantitative Determination of Human TNC

Fifty microliter aliquots of the crevicular fluid samples eluted from paper strips were serially diluted into PBS and coated onto soft 96-well dishes (Falco, BD Biosciences, Basel, Switzerland) for 1 hour. After blocking with 1% skim milk powder in PBS, wells were incubated with mouse anti-human TNC mAb B28-13 at 5 µg/mL in milk/PBS for 1 hour,[23] followed by peroxidase-labeled goat-anti-mouse IgG (Jackson Laboratories, ME, USA). Wells were developed with phenylene diamine and the optical density at 492 nm determined in an ELISA reader as published.[22] Solutions with known concentration of human TNC (obtained from medium conditioned by human IMR-90 lung fibroblasts) were used to prepare standard curves. The amount (ng) of TNC in individual samples was determined by fitting the linear range of the dilution curves to the standard curve run in parallel in each assay (Figure 2). The concentration (ng/mL) of TNC in GFC and PISF was calculated by dividing the amount of TNC (measured from ELISA) by the sample volume (determined from Periotron measurements) for each individual sample.

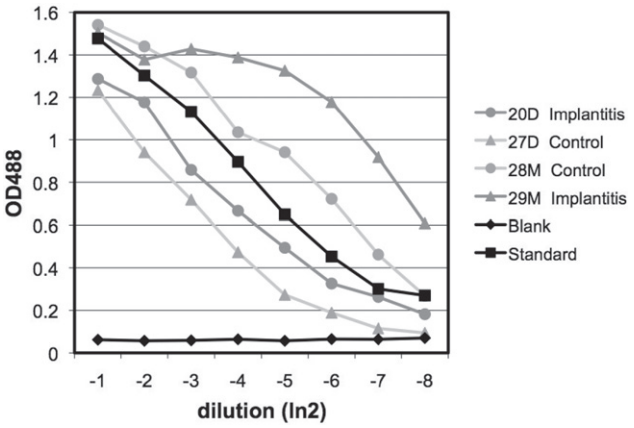

Figure 2 Example of an ELISA assay for quantification of tenascin-C in crevicular fluid from individuals with normal teeth (control) or in sulcus fluid from peri-implantitis patients (Implantitis), respectively. For each sample, a series of twofold dilutions was measured (for details, see Materials and Methods). The amount of tenascin-C in each sample was determined by fitting the linear range of the dilution curves to a standard curve obtained with known concentrations of human tenascin-C (Standard), which was run in parallel in each assay. Blank indicates a dilution curve obtained with a culture medium sample devoid of tenascin-C.

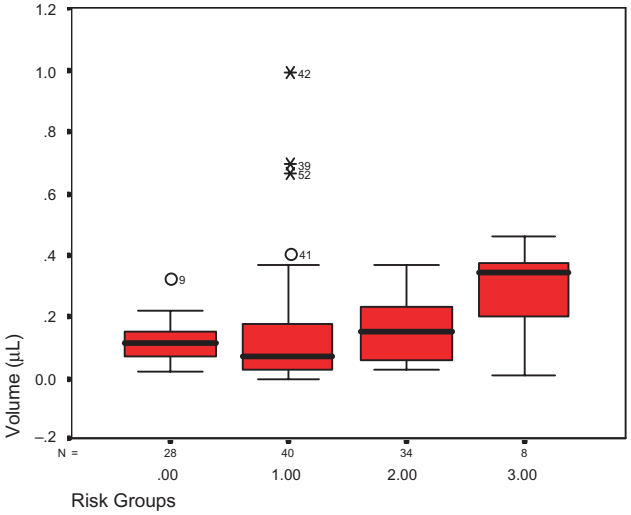

Figure 3 The range of gingival crevicular fluid/peri-implant sulcus fluid sample volume (µL) according to healthy tooth control group (0.00) and implant risk groups I (1.00), II (2.00), and III (3.00). For explanation of boxplot, see Materials and Methods.

Immunoblotting for TNC-30µL samples of GCF or PISF were run on 7.5% polyacrylamide-SDS gels under reducing conditions and blotted to nitrocellulose membrane (BA-85, Schleicher&Schuell). Blots were incubated with monoclonal antibody B28-13 against human TNC[23] (5 µg/mL) followed by horseradish peroxidase-labeled secondary antibody (Jackson Laboratories), and developed by enhanced chemoluminescence (Amersham).

Statistical Analyses

All data were processed in the computer, and statistics were performed using the SAS system program package (SAS Institute Inc., Cary, NC, USA). Means and standard deviations were compared between teeth and implant sites and within the three categories of implant sites (Mann–Whitney *U*-test).

Vertical boxplots (Figures 3–7): The boundaries of the box are Tukey's hinges. The median is identified by a horizontal line. The length of the box is the interquartile range (IQR) computed from Tukey's hinges. Values more than three IQRs from the end of a box are labeled as extremes (asterisks). Values more than 1.5 IQRs but less than three IQRs from the end of the box are labeled as outliers (O). For both extremes and outliers, the sample number is indicated.

Regression analysis (Tables 1–3) was applied with the dependent variables, BSA, MMP-9, and TNC.

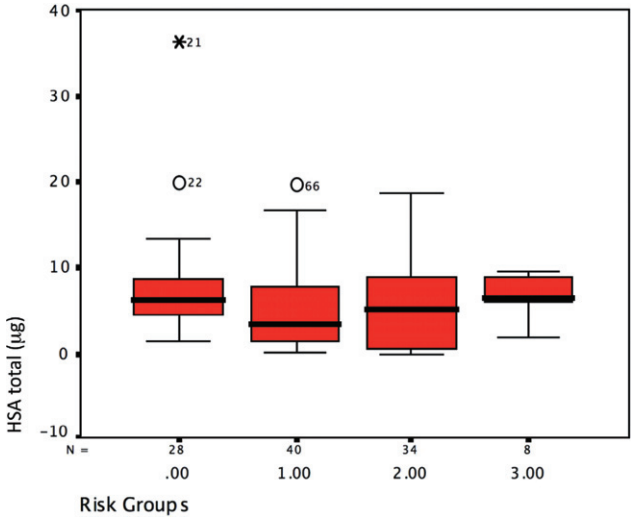

Figure 4 The amount (µg) of human serum albumin (HSA) per gingival crevicular fluid/peri-implant sulcus fluid sample according to healthy tooth control group (0.00) and implant risk groups I (1.00), II (2.00), and III (3.00). For explanation of boxplot, see Materials and Methods.

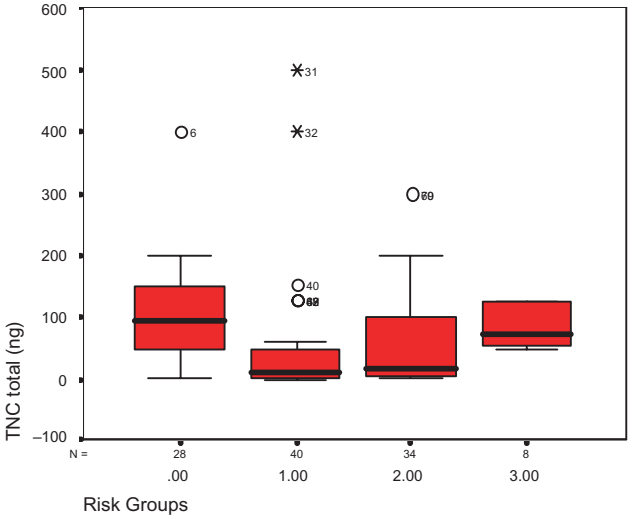

Figure 6 The amount (ng) of tenascin-C (TNC) per gingival crevicular fluid/peri-implant sulcus fluid sample according to healthy tooth control group (0.00) and implant risk groups I (1.00), II (2.00), and III (3.00). For explanation of boxplot, see Materials and Methods. Second box (1.00), lower circle: 48, 67; third box (2.00), single circle: 69, 70.

RESULTS

One hundred and ten sites from 41 implants and 14 teeth were available for measurements. Forty-one implants had successfully supported their prostheses for at least 24 months. The periodontal measurements revealed the following classification of the implant sites:

- Sites with Category I: $n = 40$
- Sites with Category II: $n = 34$
- Sites with Category III: $n = 8$

Three patients exhibited implants with sites belonging to different classifications (II and III).

At healthy control teeth, 28 sites were measured.

The range of volumes for collected GCF/PISF was comparable and large for teeth and implants. Figure 3

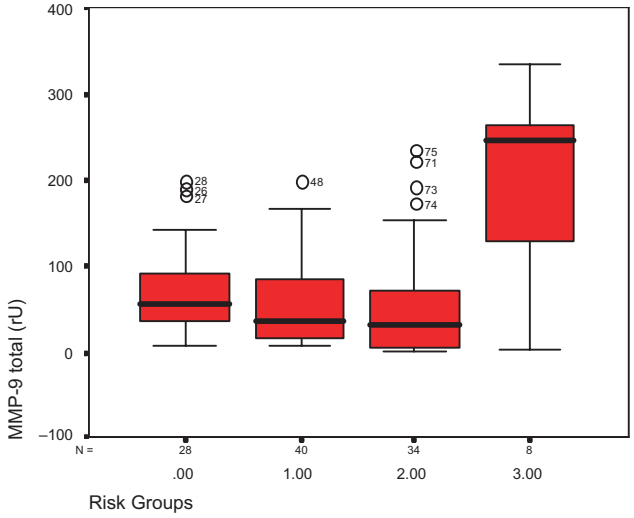

Figure 5 Metalloproteinase (MMP)-9 activity (rU, arbitrary units relative to standard) per GCF/PISF sample according to healthy tooth control group (0.00) and implant risk groups I (1.00), II (2.00), and III (3.00). For explanation of boxplot, see Materials and Methods.

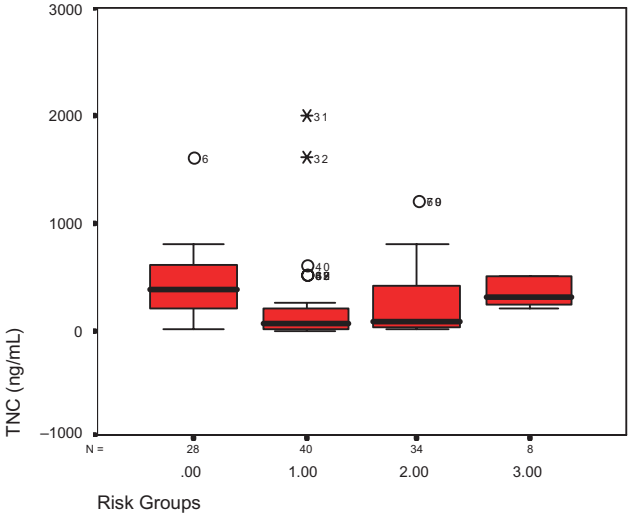

Figure 7 The concentration (ng/ml) of tenascin-C (TNC) per gingival crevicular fluid/peri-implant sulcus fluid sample according to healthy tooth control group (0.00) and implant risk groups I (1.00), II (2.00), and III (3.00). Second box (1.00), lower circle: 48, 67; third box (2.00), single circle: 69, 70.

TABLE 1 Regression Analysis with Dependent Variable Amount of HSA

Independent Variable	Mean Square	*p* Value
Teeth/Implants	94.6434216	.06
PD	4.8211951	.67
AL	14.9003837	.45
BoP+	92.2937751	.06
GCF/PISF µL	263.1599426	.002*

*$p < .05$.
AL, attachment loss; BoP, bleeding on probing; GCF, gingival crevicular fluid; HAS, human serum albumin; PD, probing depth; PISF, peri-implant sulcus fluid.

provides sample volume according to the three implant categories and the healthy teeth control. Sample volume (µL) was significantly different between healthy implant sites and sites of category II or III. It was also much higher in category III compared with the control group (teeth).

The amount of serum albumin per sample, normalized to the collection time, did not differ significantly between normal teeth, healthy implants, and sites with signs of peri-implantitis (Figure 4). Relative MMP-9 activity was increased in peri-implantitis samples when compared with the other groups (Figure 5).

The amount of TNC (ng per sample) in crevicular fluid collected from healthy teeth was significantly higher than in sulcus fluid from healthy implants. A significant difference between healthy implants (risk group I) and the peri-implantitis group (risk group III) was also observed (Figure 6). Very similar results were obtained for the concentrations of TNC (ng per mL of GCF or PISF, respectively) when they were calculated for each sample individually (Figure 7). We also analyzed by immunoblotting the molecular species of TNC in sulcus fluid. Whereas intact subunits of the human protein (large splice variant) have a size of ~250 kDa, we found that TNC in PISF was largely degraded to fragments of ~70 and ~40 kDa (Figure 8).

The results of the regression analysis (Tables 1–3) showed a significant correlation ($p = .002$) between the amount of BSA and the volume of GCF/PISF. In contrast to the amount of TNC, MMP-9 activity was significantly correlated with the PD and the volume of crevicular fluid. A highly significant correlation between the amount of active MMP-9 and PD was observed ($p < .0001$). The amount of TNC was significantly

TABLE 2 Regression Analysis with Dependent Variable Amount of MMP-9

Independent Variable	Mean Square	*p* Value
Teeth/Implants	6235.40824	.20
PD	68,899.92330	<.0001*
AL	13,576.28200	.06
BoP+	3,838.20571	.31
GCF/PISF µL	20,482.84931	.02*

*$p < .05$.
AL, attachment loss; BoP, bleeding on probing; GCF, gingival crevicular fluid; MMP-9, metalloproteinase-9; PD, probing depth; PISF, peri-implant sulcus fluid.

TABLE 3 Regression Analysis with Dependent Variable Amount of Tenascin-C

Independent Variable	Mean Square	*p* Value
Teeth/Implants	33,489.85973	.03*
PD	1,749.46126	.63
AL	6,489.55740	.35
BoP+	23,846.13842	.07
GCF/PISF µL	11,677.22206	.21

*$p < .05$.
AL, attachment loss; BoP, bleeding on probing; GCF, gingival crevicular fluid; PD, probing depth; PISF, peri-implant sulcus fluid.

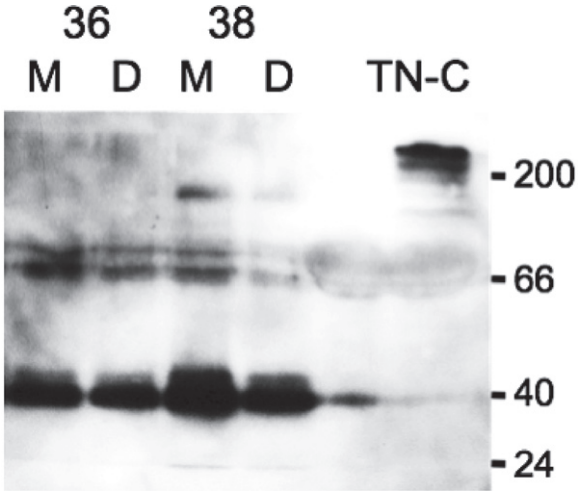

Figure 8 Immunoblot of peri-implant sulcus fluid, developed with mooclonal antibody to human tenascin-C (TNC). The numbers on top of the gel refer to two different patients; samples from mesiobuccal sites are labeled with M, and those from distobuccal sites with D. For control, the lane labeled TNC was loaded with medium from cultured human lung fibroblasts that contained secreted TNC. In contrast to the control sample, the patient samples exhibit immunoreactive fragments of various sizes but no full-size TNC subunits (250 kDa).

different for implants and teeth ($p = .03$). No correlation was observed between TNC and BoP or AL with either measurement.

DISCUSSION

The present study has measured MMP-9, has, and TNC, and has shown that they can be detected equally in the sulcus fluid of teeth and implants, with a wide individual range of volume.

Some researchers emphasized the similarity of periodontal and peri-implant tissues regarding the inflammatory process at implants and teeth.[24–28] Other researchers had some doubts as to whether the periodontal and peri-implant tissues should be considered a comparable entity regarding structure and function. The first one is a highly specialized developmental tissue, whereas the second one is a scar tissue of wound healing.[29] In the present study, the amount of volume of GCF and PISF was measured with the Periotron® 6000, which was calibrated with known volumes of buffered solution. It is debatable whether or not such volume measurements are reliable and important. In healthy sites, collecting crevicular fluid is difficult. In fact, for both implants and teeth, a slight correlation was found between the measured volume of sulcus fluid and the amount of HSA and MMP-9. However, this was not true for TNC. The sample volume was significantly higher in implant sites with signs of peri-implantitis.

HSA is the major protein species in crevicular exsudate and commonly used to determine the quantity of other proteins, by comparing an unknown quantity of protein to known amounts of HSA.[20,21] The amount of serum albumin per sample, normalized to the collection time, did not differ significantly among normal teeth, healthy implant, and peri-implantitis groups.

MMP-9 is known to be a marker of bone resorption and bone remodeling, particularly in conjunction with loosening of prostheses, such as hip, in the human body.[5,7] Relative MMP-9 activity was increased in peri-implantitis samples when compared with healthy tooth sites or implant sites with classification I or II. It might not be surprising that MMP-9 was significantly related to increased PD, which is a sign of bone loss around implants.[28]The hypothesis that concentration of MMP-9 is increased in affected sites is confirmed by the present results, and the findings are comparable with other results.[28–30]

Various studies analyzed whether measurements of BoP and PD are useful as predictors of disease activity.[31,32] However, none of these studies revealed the specificity of periodontal indices for peri-implant soft tissue based on a long-term controlled follow-up. A recent review exhibits controversy on this topic.[33] It was suggested that the absence of BoP be used as a criterion for stability rather than a predictor of disease activity.[34–36] From a clinical point of view, absence of BoP around implants would then indicate healthy peri-implant tissues. The degree of bleeding (single small spots, moderate or heavy profuse bleeding) is not discriminated by this index, but could reflect various degrees of an inflammatory status. In the present study, BoP did not reveal any association to any of the measurements. If positive BoP is an overt sign of inflammation and peri-implantitis then increased MMP-9 might be rather a marker for bone turnover and aseptic bone resorption than for an infectious process.

TNC was detected and measured in the present study at teeth and implant sites in healthy conditions and with signs of peri-implantitis. No comparable studies on such measurements are available so far. The present results indicate that TNC levels were low in sulcus fluid obtained from healthy implant sites, but a significant increase was observed in sites of classification III, indicating that this ECM protein might be of potential use as a molecular marker of bone resorption. The regression analysis did not exhibit a correlation between PD and TNC concentration. Thus, the bone resorption might be due first of all to a bone remodeling processes and not infection. Interestingly, TNC concentration was higher at teeth than at implants with a larger standard deviation. Tenascin is accumulated in the periodontal tissue of teeth, which may explain its slightly higher concentration at teeth than at implants.

TNC is known to be induced in inflammation. Contrary to our original hypothesis, however, the increase found in peri-implantitis was less than expected. On the one hand, in the context of peri-implantitis, TNC might be a marker of bone remodeling rather than inflammation. On the other hand, increased proteolysis might counteract any enhanced TNC production, as we found mainly fragments rather than intact TNC in the sulcus fluid of implant patients. A possible degradation of TNC by MMPs or other (eg, bacterial) proteases needs to be studied in the future when the process of peri-implant bone loss is investigated.

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