Peri-implant and Paracrestal Inflammatory Biomarkers at Failing Versus Surviving Implant Sites in a Beagle Dog Study

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Purpose: *This study sought to quantify three biochemical mediators of inflammation (tumor necrosis factor alpha [TNF-α], superoxide anion [SOA], and myeloperoxidase [MPO]) by analyzing crestal (peri-implants) and paracrestal gingival biopsy samples obtained from an experimental study on beagle dogs treated with implants inserted immediately into fresh sockets with circumferential defects.* ***Materials and Methods:*** *In 10 beagle dogs, 4 roughened titanium implants (3.8 mm wide × 8 mm high) were placed in the distal sockets of the third and fourth premolars, where a circumferential defect (5 mm wide and 5 mm deep) had been previously created by trephination. After varying follow-up periods, ranging from 80 to 190 days, the dogs were explored clinically to assess implant survival, peri-implant pocket depth, and implant stability. The levels of three biochemical mediators of inflammation (MPO, TNF-α, and SOA) were investigated using the crestal and paracrestal gingival biopsy samples with ELISA tests.* ***Results:*** *It was found that 37.5% of the implants were either absent or mobile. Higher levels of the inflammatory mediators were found in the crestal samples than in the paracrestal samples. The final implant stability values were significantly correlated with the final probing depth (r = –0.83, P < .01), but neither of the clinical measures were significantly correlated with any biochemical marker. The risk of implant failure was significantly proportional to the level of MPO (odds ratio: 1.1) and TNF-α (odds ratio: 1.1) in both the crestal and paracrestal regions.* ***Conclusion:*** *All the inflammatory mediators studied were higher in the crestal areas than in the paracrestal regions, but only the values of MPO and TNF-α were significant predictors of implant failure.* Int J Oral Maxillofac Implants 2017;32:807–813. doi: 10.11607/jomi.5567

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To date, the most widely accepted method for diagnosing periodontal disease or peri-implant status is based on clinical judgment after inspection of the gingival inflammation by observing bleeding upon probing of the target tissues and measuring the pocket depth. However, emerging trends in oral and periodontal diagnostic research are moving toward methods by which risk can be identified and quantified by objective measures, such as biochemical markers of disease or inflammation.[1,2]

The inflammatory response plays a fundamental role in oral surgery, being an essential process for repairing traumatized or infected tissues. This response can also serve as an early and objective means for monitoring health, by quantifying the biochemical markers of inflammation, even before clinical signs and symptoms are measurable. The repair of injury, and in particular the repair of a bone lesion around a dental implant, is a sequentially organized and coordinated mechanism of the body,[3] wherein the inflammatory response is directed to remove damaged tissue and prepare the site for either regeneration or repair.[4]

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Since dental implants emerge from the bone toward the septic oral environment, the interactions between bacteria and host cells in the peri-implant tissues result in the release of several cytokines, depending on the nature of the bacterium and idiosyncratic host immune response.[5] Several authors have found a close relationship between the degree of inflammation in peri-implant tissues and the various components collected from the peri-implant sulcus fluid (PISF), such as interleukin-1β,[6] prostaglandin E2,[7] matrix metalloproteinases,[1] myeloperoxidase (MPO),[1,2] and products of nitric oxide metabolism.[8] In addition, the level of MPO has been shown to be a good indicator of neutrophil activity in failed peri-implant sites as compared to successful endosseous dental implant sites.[1,2] Thus, MPO may be a good marker of the inflammatory process and of implant failure.[1,2] Some authors have suggested that MPO production is not confined to the inflamed sites, but also extends to sites that otherwise appear healthy.[1]

The present study sought to quantify three biochemical mediators of inflammation (MPO, tumor necrosis factor alpha [TNF-α], and superoxide anion [SOA]) by analyzing crestal (peri-implants) and paracrestal (homolateral vestibule) gingival biopsy samples obtained from an experimental study on beagle dogs treated with postextraction implants inserted into defects with healing abutments.

MATERIALS AND METHODS

Animal Experimental Phase: Surgical Management

Ten beagle dogs (5 males and 5 females), 2 to 8 years of age, without active periodontal disease were presedated by intramuscular injection using a combination of 7 mg/kg of ketamine (Parke Davis, Pfizer), 0.2 mg/kg of diazepam (Normon), and 0.05 mg/kg of atropine (B. Braun Medical SA). This was followed by laryngeal intubation and an induced general anesthesia using a continuous intravenous infusion of propofol (1 mg/kg/min). During the general anesthesia, the dogs inhaled oxygen from a ventilation tube. In addition, submucosal injections of articaine hydrochloride were infiltrated locally to control bleeding.

Full-thickness mucoperiosteal flaps were raised adjacent to the mandibular third and fourth premolars of both sides of the arch. These teeth were first sectioned horizontally at the cementoenamel junction level, and then a vertical interradicular section was performed for dividing the mesial and distal roots, avoiding root or alveolar cortical bone fracture during the extractions. This procedure was performed under copious irrigation using a thin carborundum disc mounted on a surgical handpiece.

The third and fourth mandibular premolars of both sides were extracted. In all distal sockets, a cylindrical defect of approximately 100 mm^3 was created (5-mm diameter × 5-mm depth) using a millimeter-marked trephine (229RF, JOTA AG). Two roughened titanium implants (3.8-mm diameter × 8-mm length) were then placed into the third and fourth premolar defects following the manufacturer's surgical protocol (Microdent SL). The margin of the rough-smooth junction was placed at bone level so that the polished collar emerged above the bony crest. Thus, a 0.6-mm-wide and 5-mm-deep circumferential gap was created between the implant surface and the bone wall.

After checking the primary stability of all implants, a resonance frequency analysis was conducted using an Ostell instrument (Ostell model 6.0, Integration Diagnostics) and a transducer to ensure proper implant placement. Resonance frequency was given in the form of an implant stability quotient (ISQ values) at both baseline and in the final follow-up observations.

No occlusive membranes were used over the surgical sites. Implants were then covered with a 2-mm healing abutment, and the flaps were closed with single interrupted 3-0 silk sutures, leaving implants emerging from the gingiva and exposed to the oral environment (nonsubmerged approach).

The postoperative care protocol comprised a daily dose 1 million units of benzylpenicillin (Antiobioticos Farma SA) and 3 mg/kg/d of ketoprofen (RatioPharm SA) injected intramuscularly during 4 days. Sutures were removed 1 week after surgery, and no oral hygiene protocol or soft diet was implemented during the follow-up period.

Dogs were followed up until 80 days (n = 3), 100 to 140 days (n = 3), or 160 to 190 days (n = 3) and were then explored clinically by probing the peri-implant pockets. The peri-implant pocket measurements were performed at four sites (buccal, mesial, distal, and lingual) around each fixed implant using a SM15 periodontal probe (Bontempi), recording the nearest millimeter on probing. The gingival inflammation was clinically estimated by observing the presence of bleeding around the standing implants upon probing. Implant stability, in terms of resonance frequency, was determined under general anesthesia using the already described protocol. The dogs were then euthanized at the different follow-up periods with an overdose of sodium pentobarbital (Propofol Hospira, Hospira Productos Farmacéuticos y Hospitalarios).

All procedures related to animal management were carried out according to the guidelines for animal experiments established by the Bioethical Committee of the University of Salamanca (Spain).

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Crestal and Paracrestal Gingival Sampling

The implants were considered to have failed if they were absent or mobile at the time of final evaluation. For all of the surviving implants, implant stability (ISQ values) and probing depth (mm) were assessed. Afterward, four gingival samples were obtained from the peri-implant soft tissue (crestal) and the homolateral vestibule (paracrestal) of each dog using Adson tissue forceps and a scalpel with a No. 15 surgical blade. Two samples were collected from each side of the jaw. One sample was taken from the peri-implant soft tissue in the crestal area near the gingival margin (attached gingiva), surrounding the implants at the vestibular side, and the other sample was taken from the paracrestal area, near the junction in which the attached gingiva is transformed into mobile alveolar mucosa (vestibule limit), as depicted in Fig 1. Hereafter these samples are referred to as "crestal" and "paracrestal" samples. The crestal samples were near the implants that had been inserted into the surgically created circumferential bony defect and were at risk of microorganism invasion and functional occlusal loading, whereas the paracrestal samples were not exposed to any of the aforementioned sources of inflammation (Fig 1). The dimensions of the samples were approximately 10 × 5 × 3 mm. The collected tissues were placed in sterile tubes and stored at –80°C.

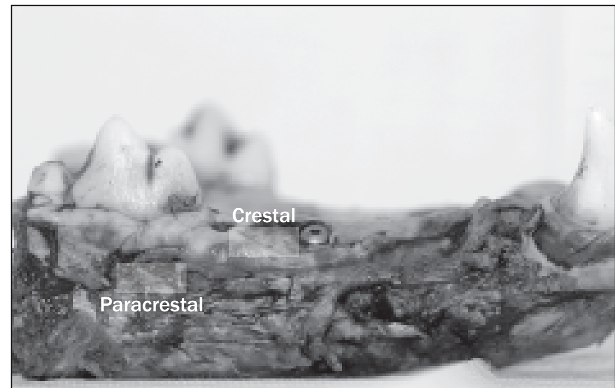

Fig 1 Diagram of the crestal and paracrestal regions in the canine jaw.

Biochemical Procedures

Three different techniques were used to obtain the values of the three biochemical mediators of inflammation investigated in this study, SOA (nmol/mg prot/min), MPO (ng/mL), and TNF-α (pg/mL). These markers were chosen because they are known to have a clear relationship with in situ inflammatory reactions, as explained below.

Superoxide anions are released by polymorphonuclear leukocytes (PMNs) to kill bacteria during microorganism invasion, and at the same time, this oxidative response contributes to maintaining inflammatory disease.[9] The presence of MPO, a neutrophil-specific enzyme, was used to evaluate neutrophil accumulation in tissues. It has been demonstrated that the level of MPO is a good indicator of peri-implant inflammation.[1,2] TNF-α has been used as a proxy of the main cytokines, since TNF-α is a cytokine that plays an important role in immune and inflammatory responses, and acts as a potent stimulator of bone resorption by promoting the degradation of the extracellular matrix.[10]

All the analytical methods used to study the inflammatory mediators were performed using standard techniques designed by experts belonging to the Experimental Surgery Laboratory of the University of Salamanca.

Evaluation of Tissue SAO Levels

The SOA levels in gingival specimens were measured in the soluble fraction of tissue extracts as previously described by García-Criado et al.[11] The protein content of each sample was determined spectrophotometrically using the Bradford assay.[12] The results were expressed as nmol/mg prot/min.

Evaluation of Tissue MPO Levels

The gingival tissue was weighed, cut into small pieces, and added to the lysis solution to a final concentration of 100 mg of tissue/mL. The tissues were homogenized on ice with a polytron homogenizer in 0.6 mL of ice-cold lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerine, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, and 28 µg/mL aprotinin [pH 7.4]). The homogenate was centrifuged at 4°C for 15 minutes at 1,500 g. The supernatant was diluted 1:5 and used to determine the concentration of MPO by means of an enzyme-linked immunosorbent assay (ELISA) (General ELISA System Technology, Menarini Labs). The results were expressed as nanograms of MPO per milliliter (ng/mL).

Evaluation of Tissue TNF-α Levels

The collected tissues were placed in sterile tubes containing 400 µL of phosphate-buffered saline and 0.05% Tween-20. All samples were stored at –20°C until used. Next, the tissue was weighed and cut into small pieces. The weight/volume ratio was of 100 mg of tissue per 1 mL of ice-cold lysis buffer. The tissues were homogenized on ice with a polytron homogenizer in 0.6 mL of ice-cold lysis buffer (same as for MPO). The homogenate was centrifuged at 4°C for 15 minutes at 1,500 g. The supernatant was collected and had a final volume of 100 mL, and was used to determine the concentration of TNF-α by means of ELISA (General ELISA System Technology, Menarini Labs). Tissue TNF-α levels were expressed as picograms per milliliter (pg/mL).

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Table 1 Description of the Study Sample

Animal-related variables (n = 10 dogs)	
Sex, n (%)	
Female	5 (50)
Male	5 (50)
Age, mean (SD), y	5.5 (3.2)
Weight, mean (SD), kg	11.6 (3.1)
Follow-up, mean (SD), days	117.7 (34.3)
Follow-up periods, n (%)	
< 80 days	3 (30)
100–140 days	3 (30)
160–190 days	4 (40)
Implant-related variables (n = 40 implants)	
Implant survival rates, n (%)	
Failed	15 (37.5)
Uneventful	25 (62.5)
Stability of implant and soft tissues, mean (SD)	
Baseline ISQ values	58.1 (5.7)
Final ISQ values	57.4 (9.7)
Final probing depth, mm	4.8 (1.8)
Bleeding on probing in standing implants, %	58.8 (50.0)

Statistical Analysis

All data were statistically analyzed with SPSS v.21 (Statistical Package for Social Sciences). The data were described using the mean and standard deviation for quantitative variables and the frequency and percentage for categorical variables. Student *t* tests and ANOVA were used to compare the means of two or more groups, respectively. To compare two or more distributions, the chi-square test was performed. The linear relationships were explored using the Pearson correlation coefficient. Furthermore, a logistic regression model was used to predict the risk of failure as a function of all the biochemical markers of the crestal and paracrestal regions.

RESULTS

Table 1 provides a description of the study sample. The 10 beagle dogs were equally distributed for both sexes and were on average 5.5 years old, weighed 11.6 kg, and were followed up during a mean of 130 ± 43.9 days. Out of the total number of implants, only 62.5% healed without any additional events, where the rest were either absent or mobile at the time of the final evaluation. The animals were followed up for either a short period of < 80 days (30% of the dogs), for a middle period of 100 to 140 days (30% of the dogs), or for a long period of > 160 days (40% of the dogs).

The initial ISQ values were 58.1 ± 5.7, and after healing these values decreased to 57.4 ± 9.7. The final probing depth around the standing fixed implants was on average 4.8 ± 1.8 mm. Most of the standing implants (58.8%) bled upon probing.

Table 2 shows a higher level of inflammatory mediators in the crestal samples than in paracrestal samples among the three follow-up groups. Furthermore, the level of MPO was significantly higher at the failing implant sites than the surrounding surviving implant regions for both short and medium follow-up periods. However, the level of TNF-α during those two periods was significantly lower in the failure sites compared to their surviving counterparts. For the long follow-up period the opposite actually occurred, and the level of TNF-α was significantly higher among the failure sites.

Table 3 depicts the linear correlation between all of the biochemical indicators of inflammation analyzed. The highest correlation was found between the crestal and paracrestal regions within a given marker (all above 0.8). Moreover, TNF-α and SOA were found to be inversely correlated in both crestal and paracrestal samples. The MPO level at the peri-implant region was directly correlated with that of TNF-α (r = 0.43) and SOA (r = 0.20) collected at the same crestal region. Furthermore, the final ISQ values were significant and inversely correlated with the final probing depth (r = –0.83, P < .01), but none of these clinical measures were significantly correlated with any of the biochemical markers.

Figure 2 shows that the risk of implant failure can be predicted when the level of MPO and TNF-α, at both crestal and paracrestal regions, is known. The level of both parameters is proportional to the odds ratio of implant failure. The regression analysis achieved a moderate but significant predictive power (Nagelkerke R^2 = 0.37).

DISCUSSION

This study analyzed the inflammatory response of the soft tissues surrounding dental implants subjected to several unfavorable conditions (ie, nonsubmerged implants inserted into fresh sockets, previously confirmed by trephine, with circumferential bone defects and without using membranes, in beagle dogs not receiving any measure of oral hygiene or soft diet). The rationale for this experimental design was to increase implant failure rates in order to obtain an adequate subsample size for comparing the level of three highly robust inflammatory mediators (MPO, TNF- α, and SOA) within the sites where implants were osseointegrated and those that were not. Both crestal and paracrestal biopsies (Fig 1) of the gingiva were collected in order to test whether the inflammatory response might extend to the soft tissue of nearby regions, as has been reported elsewhere.[1]

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Table 2 Inflammatory Mediators in the Peri-implant and Paracrestal Samples (n = 40) of Surviving and Failed Implants

Mediator	Area	Surviving implants		Failing implants	
		Mean	SD	Mean	SD
Short follow-up (< 80 days); Failure rate: 33.3%					
SOA (nmol/mg prot/min)	Crestal	22.7	6.6	29.1	8.1
	Paracrestal	18.6	4.9	25.0	7.3
MPO (ng/mL)	Crestal*	63.4	25.4	101.2	34.9
	Paracrestal*	40.4	22.0	80.9	30.6
TNF-α (pg/mL)	Crestal	100.1	10.1	91.6	7.4
	Paracrestal*	84.9	8.0	76.9	2.5
Medium follow-up (100–140 days); Failure rate: 16.7%					
SOA (nmol/mg prot/min)	Crestal*	42.1	6.7	47.1	1.0
	Paracrestal*	43.2	5.6	29.3	1.8
MPO (ng/mL)	Crestal*	67.1	25.5	114.3	3.4
	Paracrestal*	58.3	22.7	102.8	2.8
TNF-α (pg/mL)	Crestal*	93.0	12.6	73.6	2.5
	Paracrestal*	79.1	16.2	56.9	2.6
Long follow-up (160–190 days); Failure rate: 56.3%					
SOA (nmol/mg prot/min)	Crestal	21.5	9.1	27.6	9.0
	Paracrestal	17.8	11.2	27.2	13.0
MPO (ng/mL)	Crestal	67.0	8.7	65.8	4.9
	Paracrestal	48.6	15.2	58.9	11.1
TNF-α (pg/mL)	Crestal*	93.2	9.9	119.3	27.7
	Paracrestal*	81.0	10.2	97.2	12.4

*Comparisons between failed and survived implants statistically significant (*P* <.05).

Table 3 Pearson Correlation Coefficients Between the Inflammatory Mediators Within the Crestal and Paracrestal Regions

	MPO-P	MPO-C	TNF-P	TNF-C	SOA-P	SOA-C	F-ISQ	PD
MPO-P	1							
MPO-C	0.92**	1						
TNF-P	–0.34*	–0.39*	1					
TNF-C	–0.31*	0.43*	0.83**	1				
SOA-P	0.55**	0.39*	–0.19	–0.30*	1			
SOA-C	0.39*	0.20*	–0.30*	–0.30*	0.85**	1		
F-ISQ	–0.02	0.08	0.0	–0.01	0.16	0.03	1	
PPD	0.09	–0.01	0.07	0.05	–0.09	0.08	–0.83**	1

*Significance at the .05 level (bilateral).
**Significance at the .01 level (bilateral).
C = crestal; P = paracrestal; F-ISQ = final ISQ values; PPD = pocket probing depth.

To better understand why higher levels of certain biomarkers (mainly MPO but also TNF-α) might be elevated at sites where the implant failed in comparison with those sites where the implant survived, the main factors underlying the high failure rate and the high peri-implant pocket depth of the surviving implants (Table 1) observed here should be reviewed. First, an increase in bone resorption in immediate implants in beagle dog studies has been demonstrated.[13] In addition, the peri-implant defects created in fresh sockets generate higher marginal bone resorption. The ensuing inflammatory response of this additional trauma, added to the damage generated by the trephination per se, may be considered an additional risk factor leading to bone loss and peri-implant inflammation.[14] Furthermore, the flap elevation performed to carry out the trephination impairs the vascular supply to the healing site, in the initial phase of regeneration, hampering initial wound healing and possibly having a long-lasting effect on the dimensional reduction of the supporting bone and the concomitant inflammatory response.[15,16]

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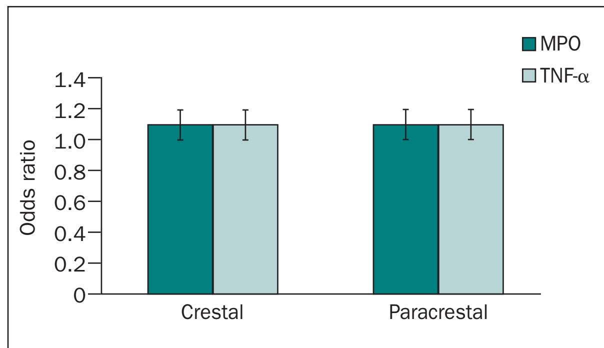

Fig 2 Odds ratio of implant failure as a function of the inflammatory mediators within the crestal and paracrestal regions after a logistic regression analysis.

It also should be taken into account that in the present study the primary stability of implants was only assured by 3 mm of the apical zone of the implant, with the rest of the implant body contained within the circumferential defect. This caused the implants to have less than optimal primary stability to absorb the indirect occlusal loading exerted toward the healing abutments. Although there is no convincing causal relation between compression and bone loss, a clear relationship between high-stressed bones and bone resorption has been found.[17,18]

Furthermore, as was the case in this study, when the wound is not protected by membranes, the mucosal opening allows the colonization of epithelial cells, which in turn induces an inflammatory response.[19] This event would then lead to fibrous encapsulation, bone resorption, and finally implant failure.[19,20]

As the dogs did not receive a soft diet during the experiment, all of the implants received indirect occlusal loads, hampering the proper osseointegration and soft tissue healing surrounding the implants.

One of the main shortcomings of the present study is that, for operational reasons, gingival samples (at both periodontal and paracrestal sites) were not collected at baseline to absolutely control intrasubject changes (before and after the intervention). However, samples were collected at two qualitatively different areas (crestal and paracrestal) to assess the potential for microorganism invasion and trauma caused by chewing. In fact, higher values were found for all of the inflammatory parameters in the crestal areas as compared to the paracrestal areas (Table 2). This result was expected, since the crestal region was at a higher risk of microorganism invasion as well as masticatory occlusal load.[17] Moreover, since all surgical procedures (extraction, trephination, and implantation) were performed within the crestal area, which is also the region with masticatory functional loads, it seems logical that this region suffered the highest and a long-lasting inflammatory response.

However, because of the design of this study, conclusions cannot be drawn regarding the cause-and-effect relationships between implant failure and inflammatory response. Although Bullon et al considered that implant failure is due to an inflammatory mechanism in which the T lymphocytes seem to play a major role,[21] it also may be plausible that the inflammatory response was a reaction to implant loss. It could be argued that T cells play an essential role in the increased levels of TNF-α at failed implant sites, as several authors have observed an increase in TNF-α levels due to a T-cell expansion associated with an estrogenic deficit.[22]

The biomarker most clearly associated with implant failure was MPO, which is a neutrophil-specific enzyme that allows the quantification of neutrophil accumulation in tissues, and is accordingly increased in infected periodontal sites[23] and in peri-implant infections.[24–26] Liskmann et al[2] concludes that elevated MPO levels were statistically associated with bleeding on probing and pocket depth around diseased and healthy implants within the same individual. Specifically, it was observed that only 9.4% of the healthy implants had MPO levels higher than 25 ng/mg, and, at the same time, 96.9% of the diseased implants had MPO levels exceeding 25 ng/mg, suggesting this level to be the cutoff point, which is in accordance with the present observations (Table 2).

Other authors have also found that the level of MPO is a better marker for inflamed peri-implant sites than nitrite-derived biomarkers.[27] Regarding the predictive value of TNF-α for failing sites, it seems to be less consistent, as only long follow-up periods revealed a positive correlation. In fact, this parameter was significantly lower at failing sites as compared with their healthy counterparts among dogs that had middle-period follow-ups of 100 to 140 days (Table 2); however, this trend was the opposite in dogs that had long-period follow-ups of 160 to190 days. This result may be explained by the fact that since TNF-α is a cytokine that promotes bone resorption, it may fluctuate according to the bone apposition/reabsorption ratio (bone remodeling). The magnitude of the bone resorption around the standing implants (subjected to chewing loads in a septic environment at baseline) is comparable to that observed within early failing sites. However, after a long period of follow-up, the level of bone resorption estimated by the TNF-α values was higher among the long-lasting failed sites in which bone lesions were more severe and more prevalent (56% of implants failed). The level of TNF-α has also been shown to be increased for a long duration during bone healing in diabetic rats.[28] Additionally, in the present study, SOA was found to be a less clear predictor of implant failure, in spite of being reported to be increased in inflamed periodontal tissues[29] or peri-implant tissues.[8] Tözüm et al also concluded that MPO is clearly a better marker of inflammation at implant sites than nitrite.[8]

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The literature does provide some evidence about the interaction of nitrite metabolism and MPO in inflammation,[30] but this is not supported by the present study's findings, probably owing to the superoxide anion short life, and the physiological fluctuations of both parameters.[31] In agreement with Boutros et al,[1] the present study found that MPO seems to be a good marker for the risk of implant failure.

Futures studies should include analyses of the peri-implant sulcus fluid, rather than the gingival biopsy samples, in order to apply a noninvasive technique for the early and objective detection of the peri-implant inflammatory process. Furthermore, the sulcus samples should be taken longitudinally to monitor biomarker changes so that the fluctuations can be analyzed together with the clinical assessments.

Another biomarker, cathepsin K, which is expressed from osteoclasts and belongs to the cysteine protease family, has been shown to be the most specific marker for ongoing resorption activity. This protein would play an important role in bone remodeling and bone destruction, since it could act as a "marker of osteoclastic activity" for both periodontal and peri-implant disease.[32]

CONCLUSIONS

All of the inflammatory mediators studied were increased in crestal areas as compared with paracrestal regions; however, only the values of MPO and TNF-α were significant predictors of implant failure.

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