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CLINICAL RESEARCH:

Oxidative Stress Markers Analysis in Sites with Healthy Conditions and with Periodontal and Peri-Implant Diseases

Análisis de los marcadores de estrés oxidativo en sitios con condiciones saludables y con enfermedades periodontales y periimplantarias

Renzo Guarnieri[1] https://orcid.org/0000-0003-1449-8340
Rodolfo Reda[2] https://orcid.org/0000-0003-1532-6524
Alessio Zanza[2] https://orcid.org/0000-0002-2062-8140
Chiara Bramucci[2] https://orcid.org/0009-0008-3270-9301
Luca Testarelli[3] https://orcid.org/0000-0003-3904-3000
Dario Di Nardo[3] https://orcid.org/0000-0002-5054-0828

[1]Private Practice, Treviso, Italy.
[2]Fondazione Policlinico Universitario Campus Bio-Medico, Rome, Italy.
[3]Department of Oral and Maxillo-Facial Sciences, Sapienza Univerisity of Rome, Italy.

Correspondence to: Rodolfo Reda - r.reda@policlinicocampus.it
Luca Testarelli - l.testarelli@unicampus.it

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ABSTRACT: To evaluate the gingival crevicular fluid (GCF) and the peri- implant crevicular fluid (PICF) concentration of oxidative stress markers, such as malondialdehyde (MDA) and superoxide dismutase (SOD) in healthy sites and in those affected by periodontitis (PER) and peri-implantitis (PIM). Sites in periodontal (PH) and peri-implant (PIH) healthy conditions and sites with PER and PIM were investigated in 120 subjects. Clinical parameters including plaque index (PI), gingival index (GI), probing pocket depth (PPD), and bleeding on probing (BOP) were recorded. Levels of MDA and SOD in GCF and PICF were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits and statistically correlated with PI, GI PPD and BOP. No statistically significant differences ($p>0.05$) in level of MDA and SOD were found in PH *vs.* PIH sites with. Compared to PH sites, PER sites presented a higher level of MDA and SOD, but the difference was not statistically significant ($p>0.05$). Statistically significant higher concentrations of MDA ($p=0.009$) and SOD ($p= 0.004$) were found in PIM sites compared to PIH. Moreover, PIM sites showed also statistically higher levels of MDA ($p=0.012$) and SOD ($p=0.013$) than PER sites. MDA and SOD concentrations were found positively correlated with PD and BOP. The measured oxidative stress markers were statistically higher in diseased peri-implant sites than in diseased periodontal sites.

KEYWORDS: Perimplantitis; Periodontitis; Oxidative stress markers.

RESUMEN: La periodontitis y la periimplantitis son enfermedades inflamatorias en las que el estrés oxidativo puede desempeñar un papel central. Este estudio evaluó los niveles de malondialdehído (MDA) y superóxido dismutasa (SOD) en el fluido crevicular gingival (GCF) y en el fluido crevicular periimplantario (PICF) de sitios sanos y enfermos. Se examinaron participantes adultos con sitios periodontales sanos (PH), periimplantarios sanos (PIH), con periodontitis (PER) o con periimplantitis (PIM). Se registraron los índices clínicos de placa (PI), gingival (GI), profundidad al sondaje (PPD) y sangrado al sondaje (BOP). Las muestras de GCF y PICF se analizaron mediante ELISA para determinar las concentraciones de MDA y SOD. Las comparaciones entre grupos y las correlaciones se evaluaron mediante ANOVA/SNK-q y las pruebas de Spearman. Se analizaron un total de 364 muestras (PH=117, PER=101, PIH=94, PIM=52) de 120 pacientes. No se encontraron diferencias significativas entre PH y PIH. En comparación con PIH, los sitios con PIM mostraron concentraciones significativamente más altas de MDA y SOD; los valores de PIM también superaron a los de PER. MDA y SOD se correlacionaron positivamente con la profundidad de sondaje y el sangrado al sondaje. Los biomarcadores de estrés oxidativo fueron significativamente más altos en los sitios periimplantarios enfermos que en los periodontales enfermos, lo que sugiere un entorno oxidativo más intenso en la periimplantitis.

PALABRAS CLAVE: Periimplantitis; Periodontitis; Marcadores de estrés oxidativo.

INTRODUCTION

Although periodontitis (PER) and peri-implantitis (PIM) are inflammatory diseases with a recognized similar polymicrobial etiology (1-4), differences in anatomic and histologic environments (5,6), core microbiomes (7), expression of mRNA for proinflammatory cytokine (8) transcripts related to the innate and bacterial immune response (9)complement activation (9), macrophage polarization (10) etc., support the hypothesis that they present a pathogenesis mediated by different interaction mechanisms between pathogenic bacteria and immune system. Moreover, some research indicated that, compared to PER, PIM shows also a more elevated inflammatory state (5,11), which could justify the greater extension and the fastest rate of progression of the lesion.

Since the oxidative stress (OS) and inflammation are closely linked (OS) can cause inflammation and this, in turn, induces OS generating a vicious circle that results in cell damage (25-27), in recent years, the determination of the OS has acquired growing attention in the evaluation of many inflammatory diseases (12-14), including the PER (15) and the PIM (16).

The term "oxidative stress" refers to the state that occurs when an imbalance in free radicals and antioxidants occurs due to the overproduction and/or mismanagement of reactive oxidant species (ROS), the loss of antioxidant defenses or both (17).

ROS (including superoxide O2-, hydroxyl OH-, hydroperoxyl HOO., nitric oxide NO., alkoxy RO-, singlet oxygen 1O2, ozone O3, hypochlorous acid HOCl, and hydrogen peroxide H2O2) (16), are highly reactive, short-lived derivatives of oxygen metabolism produced in all biologic systems and cause protein destruction, fragmentation and polymerization, lipid peroxidation, DNA mutations, and enzyme oxidation (17-20).

The harmful effects of ROS are counteracted by antioxidant defense systems, currently, classified as enzymatic [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GSR), or non-enzymatic

[glutathione (GSH), Thioredoxin reductase (TrxR), Ferritin, Transferrin, Uric acid, Ubiquinone, and Lipoic acid] (21). Oxidative stress can irreversibly damage cellular structures, including membrane lipids or lipoproteins, forming oxidation-specific epitopes on damaged cells. This damage-associated molecular pattern is recognized and removed by innate immune cells, including macrophages and neutrophils, enabling cellular homeostasis (22).

PER and PIM, like any chronic inflammatory disease, are inextricably linked with OS (23,24). Indeed, the intense recruitment of polymorphonuclear leukocytes and macrophages, through what has been defined as "respiratory burst" (25), determines in these diseases an increase in the production of ROS (26), directly responsible for the degradation of extracellular matrix components, including collagen, elastin, proteoglycans, and glycosaminoglycans (27). OS also leads to the release of lysosomal enzymes responsible for local tissue destruction. Moreover, under the influence of ROS, an increase in cytokine production and growth factors (e.g., interleukin-6 (IL-6) and -8 (IL-8), tumor necrosis factor α (TNF-α), and nuclear factor κB (NFκB)] has been shown (28). The OS can be measured in the gingival crevicular fluid (GCF) and in the peri-implant crevicular fluid (PICF) using the lipidic peroxidation final products, such as malondialdehyde (MDA), which is the most commonly studied product of polyunsaturated fatty acid peroxidation (29,30), and SOD which is an antioxidant enzyme that acts against superoxide, one of the oxygen radicals released in inflammatory pathways (31). Comparing the level of OS between healthy tissues and tissues affected by PER and PIM can provide deeper insight into the pathogenesis of these diseases. For this purpose, in the current study GCF and PICF levels of MDA and SOD were detected and compared in subjects presenting healthy periodontal and peri-implant sites and sites affected by PER and PIM.

MATERIALS AND METHODS

After approval by the Ethics Committee of the Campus Bio-Medico University of Rome, (protocol code Prot. PAR 30.21 (OSS) ComEt CBM-30/03/2021); adult subjects were selected (>18 years old), male or female, with PH, PIH, generalized PER, or PIM. All eligible individuals were invited to participate in the study and were thoroughly informed of its nature, potential risks, and benefits. The study was conducted in accordance with the Declaration of Helsinki, and participants agreeing to participate in the study signed an informed consent form. For inclusion in the study, patients had to be>18, systemically healthy, partially edentulous, with one or more missing teeth restored with fixed implant-supported restorations loaded for at least 12 months. Subjects with systemic diseases affecting the healing process (e.g., uncontrolled diabetes mellitus) were excluded from this study. Smoking subjects, patients under cancer therapy or organ transplant, pregnant or lactating women, individuals using antibiotics or immunosuppressive medication within the last three months, those needing antibiotics for infective endocarditis prophylaxis during dental procedures, those having orthodontic appliances, patients presenting oral mucosal inflammatory conditions, HIV-positive patients, and those with a history of hepatitis were excluded. Based on the effect size f=0.980 from previous studies, the one-way ANOVA method was used to calculate the sample size using G*power 3.1. One hundred- twenty participants (30 in each group) would be needed for the study at 80% power and 95% confidence level. Based on the anticipated individual variations MDA and SOD responses and the specific study design accounting for potential losses and refusals, 150 patients who had been treated with dental implants at the same office between 2010 and 2020 were selected. Thirty patients declined the invitation, bringing the total number of included patients to 120.

Each site categorized with PER, after conventional periodontal treatment of scaling and root-planning, had to present probing pocket depth (PPD) ≥ 4 mm, bleeding on probing (BOP), and radiographic marginal bone loss ≥ 3 mm (Stage III to IV). Each site categorized with PIH had to present the absence of peri-implant signs of soft tissue inflammation (redness, swelling, and BOP) and the absence of further additional bone loss following initial healing. Each site categorized with PIM had to present BOP and/or suppuration, with a probing depth (PD)>4 mm, and radiographic bone loss > 3 mm, with at least 50% of peri- implant bone remaining (otherwise, the implant was considered lost). Baseline bone level measurements on radiographs from implant surgery were reduced by 1 mm to compensate for the anticipated initial bone remodeling. If the individual had more than one implant affected by PIM and more than one tooth affected by PER only, one implant and one tooth were evaluated.

The PD (six sites per tooth), plaque index (PI), gingival index (GI), and bleeding on probing index (BOPI) index were employed to assess the periodontal clinical status. The clinical status of peri-implant tissues was evaluated by assessing the PD (six sites per implant) and corresponding PI, GI and BOP for implants. To avoid the risk of MAP and SOD fluctuation due to mechanical irritation, the clinical examination was performed a week before PICF and GCF sampling.

Calibration for clinical parameters records and MDA and SOD estimation were performed in ten sites before the actual study. Clinical recordings were done twice by a single examination within one month. The order of patients was masked and changed between the examinations. The examiner received training before the study regarding the use of each index employed for periodontal and peri-implant examinations. PD was measured using a pressure-sensitive probe (Florida Probe, Gainesville, FL, USA), and the estimation was judged to be reproducible if the agreement within ±1 mm between repeated measurements was at least 90%. The intra- examiner agreement between the two measurements was found to be 91%. A different examiner, who was blinded to the clinical records of the patients, carried out the fluid sampling.

Sites selected for PICF sampling were isolated with cotton rolls. Sample collection was performed within 30 seconds with standardized paper strips (Whatman Industries, Dartford, Kent, UK) from the buccal and lingual/palatal crevices around each implant. Then, PICF samples were placed in sterile Eppendorf tubes containing 300 µL of phosphate buffered saline (PBS). Samples were eluted for 30 minutes at room temperature before removing the Periopaper strips and then stored at -70°C until analysis.

Following the manufacturer's instructions, the MDA and SOD levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., Shanghai, China).

The MDA level in PICF was determined by the method described by Esterbauer and Cheeseman (32) based on its reaction with thiobarbituric acid (TBA) at 90-100°C and measurement of the absorbance at 532 nm. MDA reacts with TBA and produces a pink pigment which has maximum absorption at 532 nm.

SOD activity was determined by the method described by Paoletti and Mocali (33). In this method, superoxide anions are generated from oxygen molecules in the presence of EDTA$MnCl_2$ and mercaptoethanol. NAD (P) H oxidation is linked to the availability of superoxide anions in the medium. As soon as SOD is added to the assay mixture, it inhibits nucleotide oxidation. Therefore, at high concentration of the enzyme, the

absorbance at 340 nm remains unchanged. For this purpose, PICF samples were homogenized in 300 µL of phosphate buffer. After 30 minutes, the mixture was centrifuged at 4200 rpm; 400 µL of the supernatant was added to 1 mL of phosphate buffer, and was then inserted in dialysis tubes inside the phosphate buffer for 15-18 hours (4°C). The following solutions were subsequently added to the cuvette: 0.8 mL of triethanolamine-diethanolamine-HCl buffer, 40 µL of NADPH solutions, 25 µL of EDTAMnCl2 and 100 µL of different samples. In the 5th minute, mercaptoethanol was also added. Absorbance changes were detected at 340 nm.

MDA and SOD levels were considered the primary outcome and expressed in ng/ml. Data were analyzed using IBM SPSS Statistics (Version 23.0 for Windows; IBM Corp).

STATISTICAL ANALYSIS

MDA and SOD levels were considered the primary outcome and expressed in ng/ml. SPSS 11.5.0 software for Windows (IBM SPSS Statistics,Version 23.0 for Windows, IBM Corp, Chicago, IL) was used for all statistical analyses. For clinical parameters and PISF/GCF MDA and SOD levels in PH, PIH, PER and PIM the Shapiro-Wilk test was used to test the normality of the distribution. Since data were not normally distributed, the Kruskal-Wallis analysis followed by Mann-Whitney test with Bonferroni correction was performed for the comparison of noninflamed and inflamed sites. Moreover, PH, PIH, PER and PIM sites were also analyzed using the Kruskal-Wallis test, which was followed by the Mann-Whitney test with Bonferroni correction for bilateral comparisons. The correlation between MAD and SOD levels and clinical inflammatory status was analyzed with Spearman's correlation coefficient. $P<0.05$ was considered statistically significant.

RESULTS

A total of 364 GCF/PICF samples (117 in PH sites, 101 in PER sites, 94 in PIH sites, and 52 in PIM sites) collected from 120 patients were examined. Table 1 presents the demographic characteristics and the mean periodontal clinical parameters (mean± SD) of the study population. The mean age was 54.6±11.8 years. The mean number of dental implants was 2.7 (range: 1-5), and the mean number of residual teeth was 22.4 (range: 12-32). The mean PI, GI, PPD, and percentage of BOP values were significantly higher in PER/PIM sites than in healthy sites. Compared to PER, PIM sites showed significantly higher values of PD and BOP. Table 2 presents the GCF/PICF levels of MDA and SOD recorded. PH and PIH sites showed no statistically significant differences in GCF/PICF concentrations of MDA (6.86±1.06 *vs*. 7.02±1.12 ng/ml) and SOD (204.62±36.3 *vs*. 212.63±39.7 ng/ml), as indicated in Figure 1. Compared to PH sites, PER sites showed a higher concentration of MDA (9.34±1,46 ng/ml) and SOD (226.48±42.4 ng/ml), but the difference was not statistically significant ($p>0.05$). Compared to PIH sites, PIM sites showed a statistically significant higher concentration of MDA (18.32±2,78 ng/ml, $p=0.011$), and SOD (283.41±48.6 $p=0.008$). PIM sites showed also a statistically significant higher concentration of MDA and SOD than PER sites ($p=0.018$ and $p=0.015$, respectively), as underlined in Figure 2.

Spearman-rank correlation analysis showed that the PPD was positively correlated with MDA ($r=0.714$, $P=0.013$) and SOD ($r=0.808$, $P=0.004$). In addition, BOP% was positively correlated with MDA ($r=0.886$, $P=0.008$) and SOD ($r=0.821$, $P=0.009$). Other indexes were not correlated (Table 3).

Table 1. Demographic characteristics and periodontal clinical parameters (mean± SD) of the study population.

Characteristic	PH (Periodontal healthy) n. 30	PIH (Peri-implant healthy) n. 30	PER (Periodontitis) n.30	PIM (Peri-implantitis) n. 30
Gender				
Male	17 (56.6%)	19 (63.3%)	14 (46.6)	15 (50%)
Female	13 (43.4%)	11(36.6%)	16 (53.4(	15 (50%)
Age				
≤40 years	19 (63,3%)	14 (46.6%)	13 (56.6%)	12 (40%)
>40 years	11 (36.6%)	16 (53.4%)	17 (43.4%)	18 (60%)
Mean PI±SD	0.74±0.16	0.91±0.18	1.55±0.23 (*ab) a) p=0.012 b) p=0.023	1.58±0.25 (*ab) a) p=0.012 b) p=0.023
Mean GI±SD	0.64±0.18	1.05±0.21 (*a) a) p=0-024	1.54±0.17 (*ab) a) p=0.018 b) p=0.013	1.81±0.17 (*ab) a) p=0.022 b) p=0.008
Mean PPD±SD (mm)	1.62±5.4	2.05±0.17(*a) a) p=0-004	3.94±0.17 (*ab) a) p=0.013 b) p=0.011	4.87±0.19 (*abc) a) p=0.022 b) p=0.008 c) p=0.003
% BOP	12.9±5.2	14.9±6,3	62±8.1(*ab) a) p=0.007 b) p=0.005	88±11.3 (*abc) a) p=0.011 b) p=0.006 c) p=0.003

a) significant difference from PH group, b) significant difference from PIH group, c) significant difference from PER group.

Table 2. CGF/PICF concentration of MAD and SOD.

Group	n.	MPA (ng/ml)	SDO (ng/ml)
PH (Periodontal healthy)	117	6.86±1.06	204.62±36.3
PIH (Peri-implant healthy)	94	7.02±1.12	212.63±39.7
PER (Periodontitis)	101	9.34±1,46	226.48±42.4
PIM (Peri-implantitis)	52	18.32±2,78 (*abc) a) p=0.017 b) p=0.009 c) p=0.012	283.41±48.6 *(abc) a) p=0.003 b) p=0.004 c) p=0.013

a) significant difference from PH group, b) significant difference from PIH group, c) significant difference from PER group.

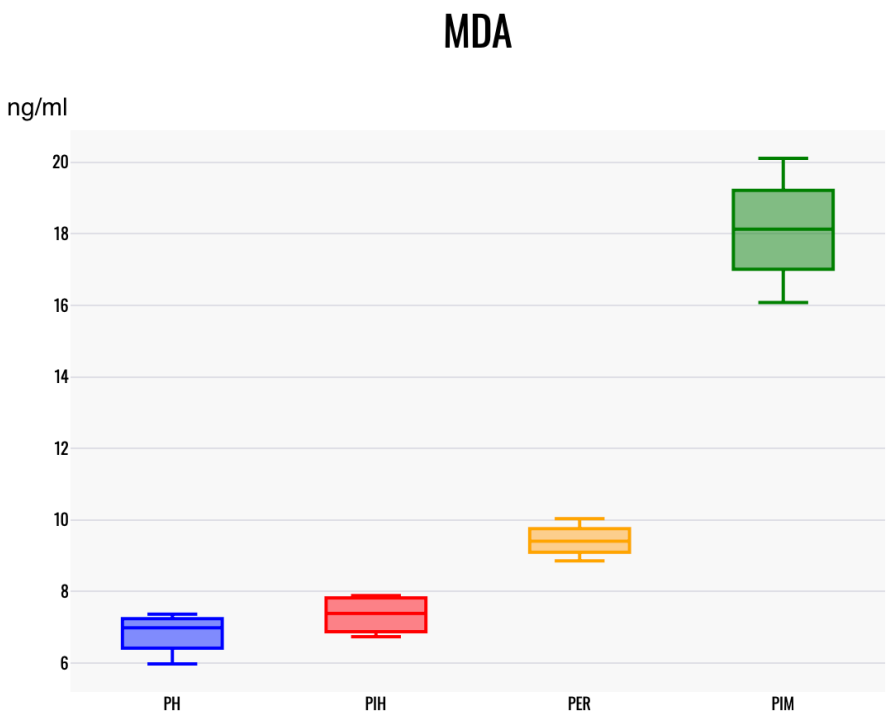

Figure 1. CGF/PICF concentration of MAD.
Statistical significance: PIM *vs.* PH p=0.011. PIM *vs.* PIH p=0.018. PIM *vs.* PER p=0.031.

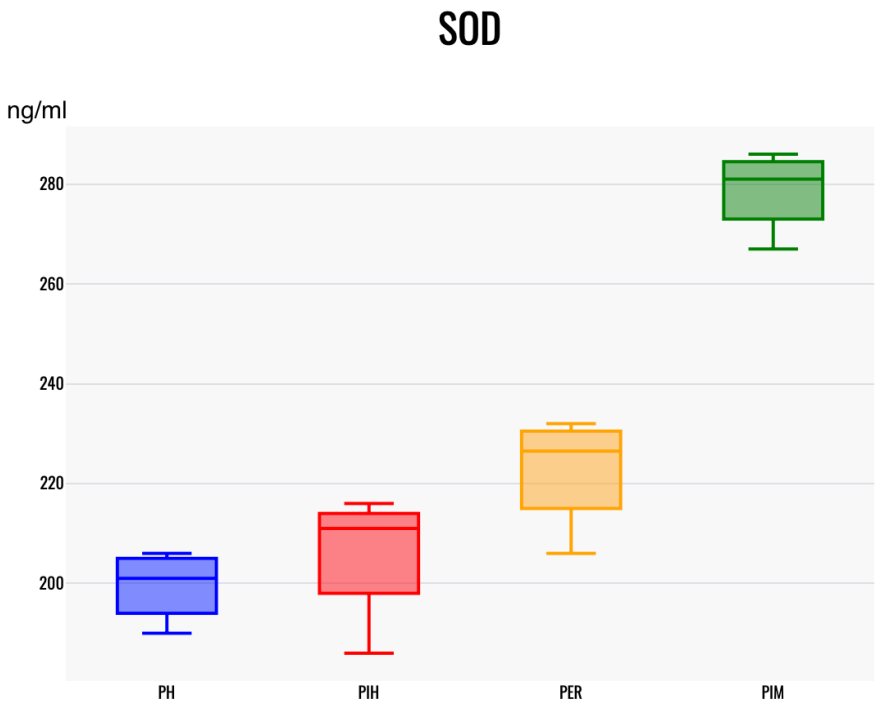

Figure 2. CGF/PICF concentration of SOD.
Statistical significance: PIM *vs.* PH p=0.008. PIM *vs.* PIH p=0.015. PIM *vs.* PER p=0.011.

Table 3. Spearman-rank correlation analysis.

Clinical parameter	MDA	SDO
PI	r = 0.127	r = 0.201
	p = 0.182	p = 0.415
GI	r = 0.164	r = 0.198
	p = 0.221	p = 0.388
PPD*	r = 0.714	r =0.808
	p = 0.013	p = 0.004
BOP*	r = 0.886	r = 0.821
	p = 0.008	p = 0.009

*Statistically positive correlation

DISCUSSION

The present study was conducted to evaluate the concentrations of MAD and SOD in the GCF and PICF of sites with PER and PIM compared to healthy controls. The objective of the evaluation was to observe possible differences in local oxidant status at sites affected by PER versus sites affected by PIM.

MDA is a well-known polyunsaturated fatty acid peroxidation product associated with the oxidation of cell membrane lipids (34). SOD is a key antioxidant metalloenzyme that play a crucial role in cellular defense mechanisms against oxidative stress, transforming superoxide anion (O2-) to continuously form H2O2 and O2 (35). In the current study, no significant differences in MDA and SDO between PH and PIH sites were found. These results indicate that in healthy condition peri-implant tissues do not differ in their state of OS compared to healthy periodontal tissues. Previous research suggested that clinically healthy soft tissues around implants may be instead characterized by a higher pro-inflammatory state, detected by a higher concentration of inflammatory cytokines, compared with clinically healthy soft tissues around teeth (36-38). This condition has been justified by the anatomical and physiological differences between periodontal and peri-implant tissues related to the interference of biomaterials during post-surgical healing. According to this report, our outcomes could suggest that the presence of OS may not be identified in peri-implant tissues not only in the presence of a health clinical state, but also in the presence of a possible subclinical state of inflammation detectable only by measuring inflammatory cytokines in the PICF. Consequently, it could be assumed that the presence of an OS state would represent a condition present only in a late phase of the peri-implant inflammation process. It is important, however, to emphasize that only two OS markers were used in the present study. Therefore, further studies using additional OS markers should be conducted to confirm our results.

The comparative analysis between PH and PER sites showed in the latter a higher MDA and SOD concentration. However, the differences did not show statistical significance. A higher OS values were instead recorded in sites affected by PER with PPD > 5 mm and high BOP indices, which were found positively correlated with GCF concentrations of MDA and SOD. Similar results have been reported by other studies (39-41) and suggest two important considerations: first, that the OS could be detectable only in the advanced stages of periodontal disease; second, that once initiated, it could cause an exacerbation of the disease itself.

However, it is important to emphasize that literature data on OS between periodontally healthy and diseased sites are conflicting, since some studies documented statistically higher levels of MAD in the GCF in tissues affected by PER compared to healthy controls (42-44). The differences reported by literature may be due to the different methods used for MAD/SOD measurement and differences in the inflammatory responses of the participants of the various studies. Therefore, further studies are needed to clarify the role of OS in PH and diseased periodontal tissues. In the current study, a higher OS was detected in PIM sites, compared to PH sites. Similar outcomes were reported also by a recent literature review in which the role of oxidative stress biomarkers in the development of peri-implant disease was summarized (17). In addition, it has been also reported that, in patients affected by peri-implantitis, the MDA and SOD concentration statistically increased also in saliva (45). An interesting finding that emerged from our analysis concerns the OS values comparation between PER and PIM sites, which showed an increased MDA and SOD concentration, statistically significant. It is known that peri-implantitis is characterized by a more severe inflammatory infiltrate compared to periodontitis induced by a preponderant innate immune response which supports a higher influx of innate and adaptive leukocytes (5,6,9). Moreover, immunohistochemical analysis suggested that at advanced peri-implantitis sites macrophages constitute a considerable proportion of the inflammatory cellular composition, with a significant higher expression for M1 pro- inflammatory phenotype, compared to anti-inflammatory M2 phenotype that mediates wound healing (10). ROS, like cytokines and commensal lipopolysaccharide (LPS), are able to stimulate M1 macrophage polarization through the activation of pro-inflammatory transcription factors, such as NF-kB and activating protein-1 (AP-1) (44-48). Moreover, a recent study on presence of ROS in inflammatory environment of peri-implantitis, documented in human peri-implant diseased tissues a high expression levels of mitochondrial genes related to ROS production (24). However, published data on relationship between MAD and SOD PICF concentration and PIM, summarizing by a recent literature review, are contrasting (16). Juan Li *et al*. (49) reported that MDA was increased in PICF of patients with peri implant disease, while Liskmann *et al*. (50) and Jazi *et al*. (51) found that there was no significant difference in MDA levels in the PICF of patients with peri implant disease compared to healthy individuals. In addition, although some studies have shown that the decreased activity of antioxidants such as SOD is closely related to peri-implant diseases, other studies have also shown that antioxidants play a protective role against free radicals in the progression of peri implant diseases. For example, Yuanke Mao *et al*. (16) found a significant increase in SOD activity in PICF of patients with peri implant disease, suggesting that SOD activity increased with the progression of inflammation. However, Liskmann *et al*. (50) found that SOD activity was reduced in patients with implant related diseases compared to healthy subjects, while Jazi *et al*. (51) reported that there was no significant difference in SOD activity between the two groups. Because of these differences, whether OS-related markers can be used as biomarkers to monitor disease development remains to be discussed. Therefore, it is urgent to identify the role of these biomarkers in the development of peri implant disease and attempt to explain the reasons for inconsistent research results.

The analysis of OS biomarkers, such as MDA and SDO, used in the present study allowed us to elucidate some possible differences between the pathogeneses of PER and PIM. However, the results must be interpreted cautiously due the listed below limitations: this study evaluated a relatively low number of subjects and biomarkers in each group; most of the study population consisted of PER-prone individuals, which may limit the generalizability of the results to a general population; due to the

cyclic progression of periodontal and peri-implant diseases, the biomarkers of immune-inflammatory events responsible for tissue breakdown may not always be detected with a single moment of fluid collection; the nonrandomized design.

CONCLUSIONS

Within the over-mentioned limits, in conclusion, results indicated that:

1. PH and PIH sites presented a same low OS.

2. PER sites presented a statistically higher OS, compared to PH, only in the presence of advanced disease.

3. Compared to PER, PIM sites presented a statistically higher OS which is independent of the severity of the peri-implant lesion.

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INSTITUTIONAL REVIEW BOARD STATEMENT: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Università Campus Bio-Medico di Roma (protocol code Prot. PAR 30.21 (OSS) ComEt CBM-30/03/2021).

INFORMED CONSENT STATEMENT: Written informed consent has been obtained from the patient(s) to publish this paper.

DATA AVAILABILITY STATEMENT: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author(s).

CONFLICT OF INTEREST: The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT: Conceptualization, R.G. and L.T.; Methodology, R.G.; Software, D.D.N.; Validation, R.R., C.B. and A.Z.; Formal analysis, R.G.; Investigation, L.T.; Resources, R.G.; Data curation, D.D.N.; Writing-original draft preparation, R.G.; Writing-review and editing, D.D.N.; Visualization, R.R.; Supervision, A.Z.; Project administration, L.T. All authors have read and agreed to the published version of the manuscript.

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