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Could Pro-Inflammatory Cytokines Levels IL-6, IL-8, TNFα, Total Antioxidant Status and Lactate Dehydrogenase Be Associated with Peri-Implant Bone Loss? A Pilot Study

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Abstract: Background: The mechanisms of periimplantitis are mostly based on biofilm-induced inflammatory lesions around dental implants. However, the host reaction, reflected by the intensity of the inflammatory response to bacterial products, is crucial for peri-implant bone destruction. The aim of this pilot study was to measure total antioxidant status (TAS), the amount of IL-6, IL-8 and TNF-α (tumor necrosis factor), salivary lactate dehydrogenase (LDH) levels and the correlation with one-year peri-implant bone loss. Methods: Seven consecutive patients (Group 1), with the presence of at least one prior inserted dental implant, and three healthy individuals, fully dentate (Group 0), were enrolled. IL-6, IL-8, TNF-α, TAS and LDH were determined in saliva of both groups. Peri-implant marginal bone loss (MBL) at one year compared to baseline was evaluated for Group 1. Results: A statistically significant positive correlation was found in Group 1 between MBL and the IL-8 ($p = 0.033$) and a strong negative correlation between MBL and TAS ($p = 0.002$), but no statistically significant differences of the assessed parameters were found between the two groups. Conclusions: In the limits of the present preliminary study, salivary TAS and proinflammatory cytokines could be associated with the risk of peri-implant bone loss over time.

Keywords: salivary biomarker; marginal bone loss; periimplantitis; total antioxidant status

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1. Introduction

Implant-anchored restorations have become, over the years, a very predictable alternative to conventional prosthetic restorations [1]. Although it is generally considered a safe, risk-free surgical procedure, with a high success rate, inserting an implant can lead to immediate failure, depending mostly on the surgical technique used, or late failure due to complications occurring over time [2]. Most of the complications can be solved without problems when diagnosed early. However, in some cases, an inflammatory process can lead to implant and bone loss or other, more severe, complications such as mandible bone fracture or oroantral communications [3,4].

Peri-implant inflammation manifests itself as peri-implant mucositis, a reversible inflammation of peri-implant soft tissues around a functional dental implant with bleeding

on gentle probing, increased probing depth, erythema and/or suppuration and periimplantitis, with additional irreversible loss of hard and soft tissues around the implants, recession of the mucosal margins and radiographic detectable bone loss [5–7].

The mechanisms of peri-implant mucositis and periimplantitis are mostly based on biofilm-induced inflammatory lesions around dental implants. The host reaction, reflected by the intensity of the inflammatory response to bacterial products, is crucial in the peri-implant bone destruction. Weyant and Burt [8] examined the survival rate of implants and observed a small number of patients losing many implants. Based on this finding, they estimated that patients who had one implant lost were 1.3 times more likely to lose more implants. This observation led to the hypothesis that host factors affect implant survival, and therefore, genetic predisposition may be involved in the development of periimplantitis. Most of the recent studies highlighted that the polymorphisms of cytokines play a vital role in the immune response, but no decisive results have been shown to recommend genetic testing prior to implants' insertion [9,10].

Cytokines are, however, considered as markers of inflammation, with the immune response to plaque pathogens being regulated by the balance between pro- and anti-inflammatory cytokines [11]. These mediators are also involved in modulating osteoclastogenesis and maintaining bone homeostasis by regulating osteoclast and osteoblast differentiation and activation [12]. Their identification and dosage could, therefore, predict peri-implant bone remodeling.

As oxidative stress and inflammatory processes lead to tissue destruction, and extracelular lactate dehydrogenase (LDH) is a marker of cell death and tissue degradation, its level in saliva is a useful biomarker for periodontal disease diagnosis and monitoring [13].

The increased oxygen consumption in the inflammatory process will determine the overproduction of free radicals, leading to the damage of oral tissue cells. Normal saliva contains enzymatic and non-enzymatic antioxidants for neutralizing the free radicals, and their concentration is claimed to be significantly decreased in periodontal patients compared to healthy individuals [14–16].

Due to its great accessibility, requiring non-invasive collection methods, saliva is a very good tool in assessing and diagnosing different oral and systemic diseases, has high availability, is painless and does not require special equipment for collection [17].

There are several studies investigating the salivary concentration of cytokines in the cases of periimplantitis [5,18,19]. However, studies predicting disease, mainly marginal bone loss (MBL), an essential component of prevention, are scarce.

The aim of this pilot study was to measure total antioxidant status, the amount of IL-6, IL-8 and TNF-α (tumor necrosis factor), salivary lactate dehydrogenase levels and the correlation with one-year peri-implant bone loss.

2. Materials and Methods

2.1. Study Design and Patient Selection

The present pilot study was conducted in accordance with the ethical principles including the World Medical Association Declaration of Helsinki, the Belmont report, the Council for International Organizations of Medical Sciences (CIOMS) guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP). The study was approved by the Bioethical Committee of "Carol Davila" University of Medicine and Pharmacy Romania, no. 176/2018. The procedure was explained, and written consent of each subject was obtained.

Seven consecutive patients (Group 1) aged between 28 and 46 years, with at least twenty teeth, none of them with untreated periapical lesions visible on radiographs, without periodontal surgery in the last six months, and three healthy individuals between 25 and 44 years old, fully dentate, without dental caries or periodontal lesions (Group 0—reference), all of them being nonsmokers, were enrolled. Before enrollment, the periodontal status of all participants was clinically assessed, and only those with absence of visual signs of inflammation and bleeding on probing [20] were included.

The mandatory criteria for the inclusion of patients in Group 1 were the following: the presence of at least one AlphaBio SPI (Alpha-Bio Tec, Petah Tikvah, Israel) dental implant inserted, loaded with a correctly executed dental prostheses for more than twelve months before enrolling in the present study; clinically healthy mucosa, no clinical or radiological signs of peri-implant/periodontal inflammation; without the presence of stomatitis or other pathological lesions; nonsmokers; good systemic health with no immunosuppressants, no medications that could affect salivary secretion nor the use of corticosteroids as medication; no antibiotic nor antifungal treatments in the past 3 months.

All included implants were inserted and restored by a single experienced implantologist (S.D.) using a strict protocol, according to the manufacturer's instructions. In brief, each patient underwent preoperative cone beam computed tomography (CBCT) scanning prior to implant insertion. After local anesthesia, the full-thickness flap was elevated to obtain direct visual access to the residual bone and the osteotomies were performed under cold saline irrigation, using a freehand technique. After insertion, all implants were covered with cover screws and then the flap was sutured with nonabsorbable interrupted sutures, and patients received written hygiene recommendations, including Chlorhexidine 0.2% and a prescription for prophylactic antibiotics (amoxicillin and clavulanic acid 1 g/every 12 h), in addition to an anti-inflammatory (ibuprofen 600 mg/every 8 h) for 5 days. Implants were exposed after 4 months (Type 4C—Late Placement and Conventional Loading according to Gallucci et al.'s classification [21]) and healing abutments were inserted. Two weeks after healing abutment insertion, the prosthetic procedure was initiated. Prefabricated titanium abutments and cemented metal-ceramic crowns were used to restore the inserted implants.

Saliva collection was performed for Group 0 (n = 3, reference group) and Group 1 (n = 7) on the same morning, without external stimuli 90 min before collection (unstimulated sample), by a single investigator (S.D.), following the same protocol. From each patient, 3 mL of saliva was collected from the floor of the mouth using a 5 mL sterile syringe. The saliva was then placed in Falcon-type millimeter tubes (Merck KGaA, Darmstadt, Germany), coded and stored at a temperature of −18 °C for a maximum of four hours until being analyzed by an investigator (D.G.) who was unaware of the identity of the samples. On the same day, the patients in Group 1 underwent radiographic examination. Digital periapical radiographs of the inserted dental implants, using individualized holders to ensure repeatability, were performed.

For Group 1, saliva collection and digital periapical radiographs were performed at 12 months post-prosthetic insertion follow-up, which is considered the baseline for the present pilot study.

In the saliva of Group 0 and Group 1, the concentration of pro-inflammatory cytokines (IL-8, IL-6, TNFα), total antioxidant activity (TAS), as well as the level of lactate dehydrogenase (LDH—marker of tissue degradation) were determined. All determinations were performed in triplicate.

The patients were instructed to perform adequate oral hygiene, including for the inserted implants. No additional professional cleaning nor monitoring was performed before the next one-year follow-up visit. The pilot study protocol is presented in Figure 1.

2.2. Determination of Salivary Cytokines IL-6, IL-8 and TNF-α

The Invitrogen™ Ultrasensitive Human Interleukin-6 (HU IL-6), Invitrogen™ Ultrasensitive Human Interleukin-8 (HU IL-8) and Invitrogen™ Ultrasensitive Human TNF-α (HU TNF-α) ELISA kits were purchased from ThermoFisher Scientific (Waltham, MA, USA). Samples were introduced into the wells to which the second monoclonal antibody was added. After the first incubation, the HU IL-6, HU IL-8 or HU TNF-α antigen binds simultaneously to the immobilized antibody and the solution. After removing the excess of the second antibody, an enzyme, streptavidin peroxidase, was added. This is used to bind the bio-initiated antibody. After a second incubation and removal of the uncoupled enzyme, a substrate solution was added. It binds to the coupled enzyme and thus produces color. A Colorimetric Microplate Reader (ThermoFisher Scientific, Waltham, MA, USA)

was used to obtain the results. The color intensity is directly proportional to the IL-6, IL-8 and TNF-α concentrations of the saliva sample.

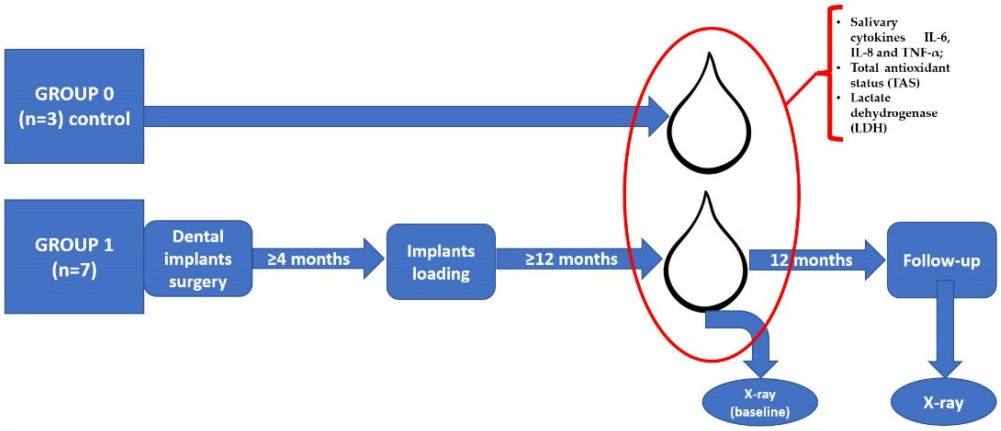

Figure 1. Pilot study workflow.

All components of the kit, including the plates used, were brought to room temperature before opening the testing kit. All saliva samples were collected in sterile tubes and frozen until laboratory analysis. Samples smaller than 500 pg/mL were diluted with ELISA Assay Buffer (ThermoFisher Scientific, Waltham, MA, USA). Since the stabilized chromogen is affected by light, its exposure to light was avoided.

Each sample was tested in triplicate. Preparation of salivary samples was performed as follows: A standard at 2500 pg/mL, using ELISA Assay Buffer, was established. Each sample was stirred, then left for 10 min to stabilize, and 0.200 mL was added in a tube with 0.800 mL of ELISA Assay Buffer. The tube was labeled with 500 pg/mL HU IL-6, HU IL-8 or HU TNF-α respectively, and 0.300 mL of ELISA Assay Buffer was added to each of the 6 wells, labeled as: 250, 125, 62.5, 31.2, 15.6 and 7.8 pg/mL. The Streptavidin-HRP100X (ThermoFisher Scientific, Waltham, MA, USA) was concentrated in 50% glycerol viscous solution. Therefore, to ensure dilution, the solution needs to be homogenized at room temperature before its use.

2.3. Total Antioxidant Status Assessment

The total antioxidant status, evaluating the susceptibility to oxidative damage, was determined with the total antioxidant status (TAS) kit, Randox Laboratories (Crumlin, UK), using a colorimetric method. The chromogen (2,2′-azino-bis-[3-ethylbezothiazoline sulfonate]), ABTS, was incubated with a peroxidase (metmyoglobin) and hydrogen peroxide, resulting in cationic chromogen radicals, $ABTS^{\bullet -}$. They have a relatively stable blue-green color, measured at 600–660 nm. The antioxidants in the test sample are responsible for the attenuation of the color, to a degree that is proportional to their concentration [22,23]. Calibration was performed with 6-hydroxy-2,5,8-tetra-methylchroman-2-carboxylic acid (Trolox®, a water-soluble vitamin E analog, Hoffman-LaRoche, Basel, Switzerland). The results are expressed in mmol/L of Trolox equivalent and described as "Trolox® Equivalent Antioxidant Capacity" (TEAC) [22]. The kit reagents were prepared according to the manufacturer's instructions. The test was performed on the culture plate. The chromogen, blank (B), standard (S) and test samples were distributed in the wells of the culture plate. They were well-homogenized, incubated at 37 °C and the initial absorbance was read as optical density (OD) at 0 min. Then, the substrate (stabilized hydrogen peroxide) was added, mixed and incubated for 3 min at 37 °C. The absorbance (OD 3 min) was immediately read at 620 nm on a Multiskan™ FC Microplate Photometer (ThermoFisher Scientific, Waltham, MA, USA). Reaction time with the substrate and temperature are key elements for a correct measurement.

2.4. Lactate Dehydrogenase Assessment

Lactate dehydrogenase in saliva was determined based on a colorimetric method, by reading the optical density (OD), using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit from Promega (Promega, Milano, Italy). Briefly, saliva was diluted 1:1 with phosphate buffered saline, and 50 µL of the diluted saliva was distributed into the 96-well flat-bottomed culture plate, over which 50 µL of the substrate provided in the kit was added. The reaction was allowed to proceed for 30 min in the dark, at room temperature, after which it was quenched with 50 µL of the stop solution from the kit. The background control sample (B) contained 50 µL of saline phosphate buffer. LDH in saliva samples was measured with a 30 min coupled enzymatic assay, which results in the conversion of a tetrazolium salt (iodonitrotetrazolium-violet) into a red formazan product. The samples were immediately read on a Multiskan™ FC Microplate Photometer at 490 nm.

LDH is expressed in relative luminescence units (RLU) calculated according to the producer's recommendation, as follows:

$$\text{LDH (RLU)} = \text{OD}_{490\,\text{nm}} - \text{B (Background)}.$$

2.5. Peri-Implant Marginal Bone Loss

Peri-implant marginal bone level was measured by an independent investigator (C.M.C.), at mesial and distal sites, on the digital periapical radiograph at the day of saliva collection and at one-year follow-up. Before any radiological examination, a film holder was directly customized in the patient's mouth using auto-polymerizing acrylic resin to reproduce the same position of the film, thereby obtaining superimposable dental radiographs at different time intervals. Bone level was measured on digital radiographs using Image J open software (imagej.nih.gov, accessed on 28 July 2021), calibrated for each measurement based on the known implant length [24]. For calibration with the known implant length, the reference points were implant apex and implant collar, and for mesial and distal bone level measurements, the reference points were the correspondent horizontal point of the implant apex and the most coronal bone to implant contact. Bone level on the first retro alveolar radiograph was considered the baseline. Bone loss at one-year follow-up was measured by subtracting the measured bone level from the baseline for each site. The mean value for mesial and distal sites was recorded (Figure 2). The formula used for the mean MBL calculation was:

$$\text{Mean MBL} = [(h0M - h1M) + (h0D - h1D)] \div 2$$

where h0M and h0D = mesial and distal bone level at T0 (same day as saliva collection), and h1M and h1D = mesial and distal bone level at T1 (one-year follow-up) (Figure 2).

For patients with multiple implants inserted fulfilling the inclusion criteria, only one implant, with the highest MBL, was considered for statistical analysis.

2.6. Statistical Analysis

All data were synthetized in Excel tables, and compared and analyzed. Origin Lab Pro 2021 (OriginLab Corporation, Northampton, MA, USA) was used for statistical analysis. The normality condition of variables was verified using the Shapiro–Wilk test. Due to the lack of normal distribution and the small sample size, the Kendall-τ (tau) test was used to assess the correlation between one-year peri-implant bone loss (MBL) and the amount of IL-6, IL-8 and TNF-α (tumor necrosis factor), total antioxidant activity (TAS) and salivary lactate dehydrogenase levels (LDH). Significance was set at a p-value < 0.05.

The nonparametric Mann–Whitney U test was used to compare cytokines values, TAS and LDH between Group 1 and Group 0 (reference).

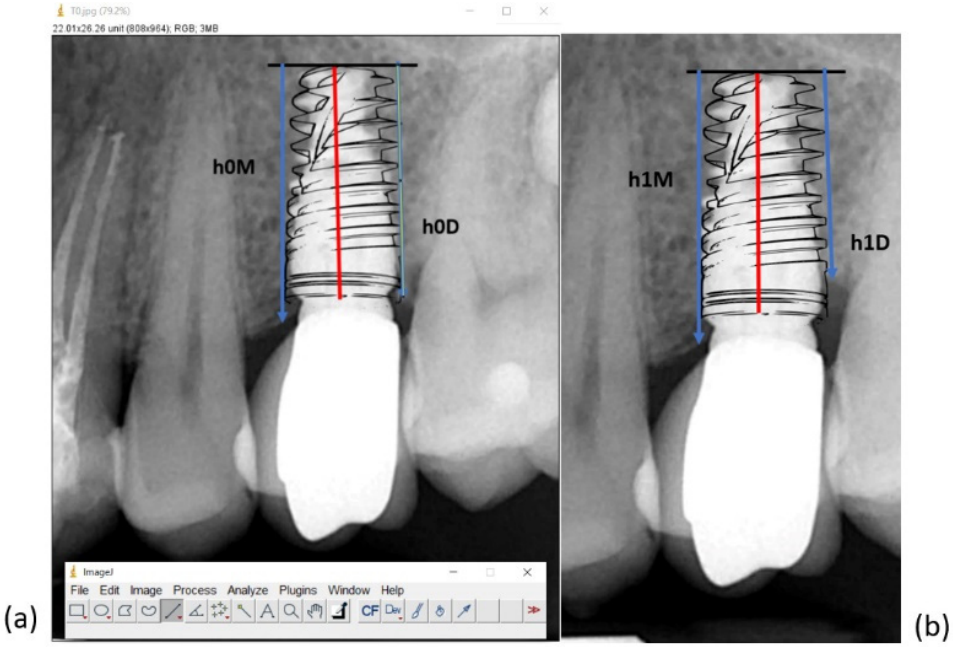

Figure 2. Marginal bone loss calculation taking into consideration: (**a**) mesial and distal bone level at T0 (h0M, ho0D), measured with Image J software, and (**b**) mesial and distal bone level at T1 (h1M, h1D).

3. Results

Among the seven patients enrolled in Group 1, four were female and three males, with a mean age (±SD) of 35.71 (±6.07). Group 0 (reference) comprised two females and one male, with a mean age of 35.33 (±9.61).

The values of the parameters assessed in the Group 1 patient saliva (IL-6, IL-8, TNF-α, antioxidant status and LDH) were compared to the mean values of the reference Group 0.

3.1. Salivary Cytokines IL-6, IL-8 and TNF-α

Salivary cytokines in patients included in Group 1 compared to the mean values of healthy persons (Group 0) are presented in Table 1. No statistical significance was found between Group 1 and Group 0 in the level of assessed cytokines.

Table 1. Salivary cytokines in pg/mL of patients included in Group 1 compared to the mean values of the reference group (Group 0).

pg/mL	Group 1 (n = 7)								Group 0 (n = 3) Mean (±SD)	p-Value
	1	2	3	4	5	6	7	Mean (±SD)		
IL-6	0.21	0.15	0.14	7.99	0.17	1.95	2.45	1.87 (±2.87)	0.52 (±0.09)	0.82
IL-8	140.43	56.47	154.52	214.75	95.91	121.22	209.62	141.85 (±57.58)	101.37 (±3.21)	0.36
TNFα	0.70	0.21	1.46	4.21	1.21	4.34	7.44	2.80 (±2.62)	2.32 (±0.04)	0.82

$p < 0.05$—statistically significant.

3.2. Total Antioxidant Status (TAS)

The TAS was determined from the manufacturer's formula using the values from ELISA. The units of measurement were mMol/L of Trolox equivalent. The values from Group 1 and the mean values from Group 0 are displayed in Table 2. No statistical significance was found between Group 1 and Group 0 in the total antioxidant status.

Table 2. Total antioxidant status (TAS) in mMol/L of Trolox equivalent for patients included in Group 1 compared to the mean values of the reference group (Group 0).

mMol/L	Group 1								Group 0 Mean (±SD)	*p*-Value
	1	2	3	4	5	6	7	Mean (±SD)		
TAS	1.20	1.25	1.09	0.61	1.60	1.80	0.72	1.18 (±0.43)	1.15 (±0.06)	1.0

$p < 0.05$—statistically significant.

3.3. Lactate Dehydrogenase Assessment

All ten saliva samples from Group 1 (n = 7) and Group 0 (n = 3) were examined in parallel, according to the producer's recommendation, less than four hours before their collection, and the half-life of LDH released from cells into the surrounding medium was approximately 9 h [25]. The LDH values expressed in RLU are displayed in Table 3. No statistical significance was found between Group 1 and Group 0 in relation to LDH values.

Table 3. Lactate dehydrogenase (LDH) expressed in relative luminescence units (RLU) for patients included in Group 1 compared to the mean values of the reference group (Group 0).

	Group 1								Group 0 Mean (±SD)	*p*-Value
	1	2	3	4	5	6	7	Mean (±SD)		
OD 490 nm	0.119	0.125	0.067	0.323	0.068	0.267	0.704	0.239 (±0.23)	0.206 (±0.01)	
Background (B)-control	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066	
LDH (RLU = OD-B)	0.053	0.059	0.001	0.257	0.002	0.201	0.638	0.173 (±0.23)	0.140 (±0.01)	0.82

OD = optical density; $p < 0.05$—statistically significant.

All evaluated patients from Group 1 and reference Group 0 had similar mean values of proinflammatory cytokines (IL-6, IL-8 and TNF-α), similar total antioxidant status (TAS) and similar levels of lactate dehydrogenase (LDH), with no statistical significance between groups. However, at a closer look (Figure 3 and Tables 1–3), patient 4 from Group 1 registered the highest level of IL-6 and IL-8, patient 7 from Group 1 registered the highest level of TNF-α and both above-mentioned patients registered the lowest level of TAS in saliva.

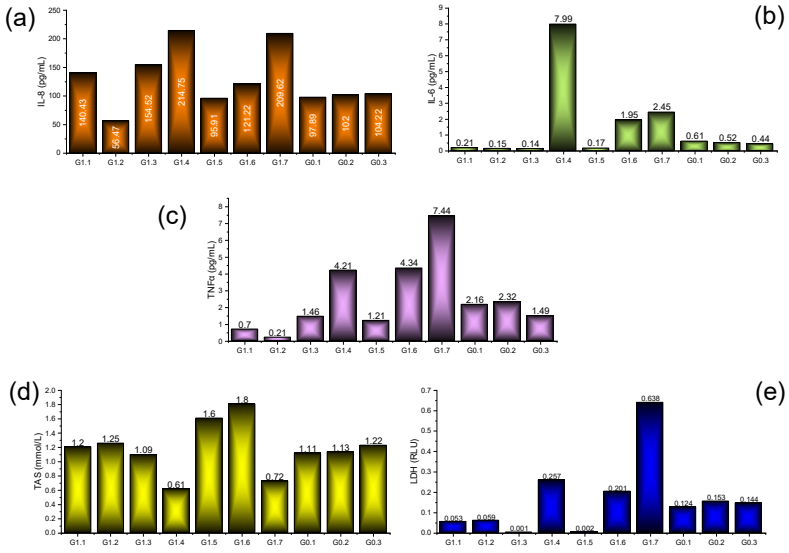

Figure 3. Proinflammatory cytokines IL-6 (**a**), IL-8 (**b**) and TNF-α (**c**), total antioxidant status (TAS) (**d**) and lactate dehydrogenase (LDH) values (**e**) for the ten patients from Group 1 (G1.1–G1.7) and Group 0 (G0.1–G0.3).

3.4. Peri-Implant Marginal Bone Loss at One-Year Follow-Up

As it could be noticed from Table 1, for most of the analyzed cases (5 out of 7 patients), the measured one-year bone remodeling was between 0.02 and 0.08 mm (Table 4). However, patients 4 and 7 registered a significant amount of bone loss: 1.0 and 0.85 mm, respectively. The two above-mentioned patients also registered the lowest values of TAS, according to Table 2.

Table 4. Peri-implant marginal bone loss (MBL) measured between baseline (same day as saliva collection) and one-year follow-up.

mm	Group 1							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**Mean (±SD)**
Mean MBL (±SD)	0.07 (±0.01)	0.07 (±0.04)	0.08 (±0.01)	1.0 (±0.17)	0.05 (±0.02)	0.02 (±0.01)	0.85 (±0.09)	0.32 (±0.45)

A statistically significant positive correlation was found in Group 1 between MBL and the IL-8 (Kendall-τ = 0.683, $p = 0.033$) and a strong negative correlation was found between MBL and TAS (Kendall-τ = −0.975, $p = 0.002$) (Figure 4).

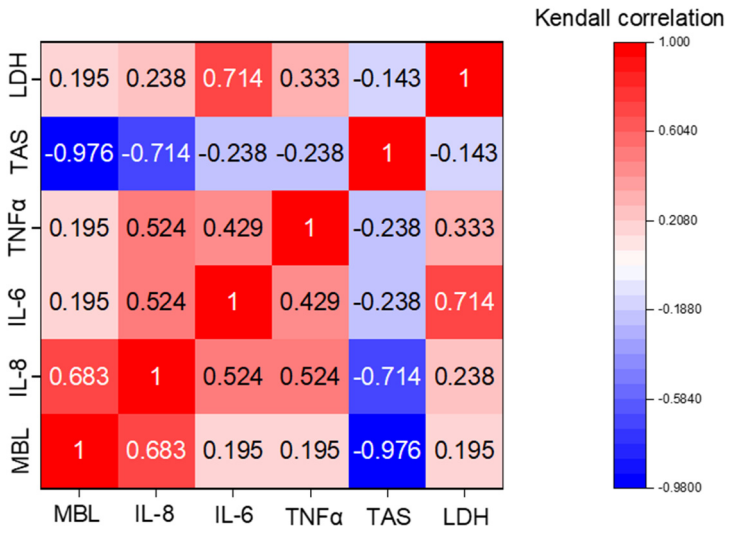

Figure 4. Analysis of correlation between one-year peri-implant bone loss (MBL) and the amount of IL-6, IL-8 and TNF-α (tumor necrosis factor), total antioxidant activity (TAS) and salivary lactate dehydrogenase levels (LDH) for patients included in Group 1. The positive correlation is displayed in red and the negative correlation in blue.

4. Discussion

In recent years, several authors [6,26–28] have focused their research on the diagnosis of peri-implant disease, its evolution, treatment and comparison to periodontal disease. Thus, various studies have been conducted [29–31] on immunological markers in saliva, oxidative stress [16], the volume and composition of the peri-implant crevicular fluid (PCF), the oral microbiota and its influence on the peri-implant/periodontal condition and disease progression.

Despite the promising results and the advantages of using salivary biomarkers, studies evaluating the prognosis of bone resorption/remodeling around implants with the aim of predicting the risky patients, prior to the occurrence of inflammatory disease, are scarce. Therefore, the aim of the present pilot study was to evaluate some proinflammatory cytokines, the total antioxidant status and LDH in saliva to see if the level of such parameters could predict bone loss around osseointegrated implants.

Moreover, due to the lack of periodontal ligaments, acting as a cellular and vascular network, comprising mesenchymal cells of diverse differentiation levels, epithelial cell rests of Malassez, along with neural and inflammatory cells, serving as defense and

repair mechanisms, the dental implants are more prone to more rapid progression of bone resorption, compared to natural teeth [32,33]. Therefore, an early diagnosis, or even better a warning in vulnerable patients who need to be closely monitored, is required. Additionally, scientific evidence for a specific bacterial biofilm composition responsible for the onset and progression of periimplantitis could not be proven. The installation of periimplantitis might depend on risk factors and the systemic condition [34], and therefore an early diagnosis of a predisposition "pattern" is more than welcome.

Lately, saliva has become the focus as a noninvasive diagnostic fluid for oral and systemic diseases, due to its great accessibility and its non-invasive, easy and low-cost collection [19]. Many inflammatory mediators associated with peri-implant mucositis and periimplantitis, such as IL-6, IL-8, MMP-8 (matrix metalloproteinase 8), IL-1β and TNF-α, have been identified as potential biomarkers [35].

Liskmann et al. [36] analyzed the utility of cytokines such as interleukin IL-6 and IL-10 as markers for periimplantitis. Interleukins are multifunctional proteins and glycoproteins that control intercellular relationships both locally and systemically. They are produced by immunocompetent cells such as T cells, monocytes or inflammatory infiltrates. The samples were obtained from fully edentulous patients, with at least two functional implants inserted in the mandible, but also from clinically healthy patients (control group). The results showed that there is a large difference between IL-6 and IL-10 concentrations in patients with periimplantitis compared to clinically healthy patients. Some clinically healthy patients have slightly elevated levels of IL-6. In contrast, IL-10 has not been found in clinically healthy patients, as it is only found in patients with periimplantitis. Similar to the authors' findings, in our study, the highest level of IL-6 was observed in the two patients with the greater MBL loss at one-year follow-up.

Konttinen et al. [37], evaluating peri-implant and gingival samples of patients with failing implants, severe periodontal disease and healthy controls, for TNF-α, IL-1α, IL-6, platelet-derived growth factor A (PDGF-A) and transforming growth factor alpha (TGF-α), reported much higher levels of TNF-α, IL-1α and IL-6 in patients with periimplantitis and periodontal compromised patients compared to healthy patients.

Campos et al. [10] analyzed the single nucleotide polymorphisms in the IL-2 gene at position −330 and the IL-6 gene at position −174 in 34 patients with failing implants and 40 patients with healthy implants. The analysis established that these genes do not pose a genetic risk and cannot be correlated with early implant failure.

Nowzari and co-workers [38] studied the levels of cytokines (IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70) in the gingival crevicular fluid (GCF) in relation to subgingival bacterial species, for both healthy teeth and implants. The results showed an increase in all cytokines in implants and teeth with high bacterial load. However, inflammatory cytokines (especially IL-1β and TNF-α) were produced even in sites with minimal bacterial accumulation.

The in vitro study by Bordin et al. evaluated the fibroblasts of patients with periimplantitis, marginal periodontitis and healthy patients. The results showed that fibroblasts, in patients with periimplantitis and marginal periodontitis, had a pronounced proinflammatory profile and synthesized higher amounts of IL-6 and IL-8 [39].

Three cytokines were selected to be assessed in the present pilot study: IL-6, IL-8 and TNF-α.

IL-6 is a proinflammatory cytokine with a central role in host defense, being produced by many cell types, such as monocytes, macrophages, fibroblasts, endothelial cells, T cells and mast cells [40]. IL-6 plays an important role in T-lymphocyte proliferation, B-lymphocyte differentiation and complement cascade activation, and in addition, can induce bone resorption [10]. Its salivary value is significantly higher in patients diagnosed with periimplantitis or periodontal disease compared to healthy controls [40–42].

IL-8 is a pro-inflammatory chemokine that may be produced by macrophages, epithelial cells, mesenchymal stem cells (MSCs), mast cells and endothelial cells, near the surface of titanium implants [43,44]. Its presence was established in periprosthetic tissues with

implant debris and was confirmed as a biomarker of peri-implant osteolysis. Moreover, implant debris can induce the production of IL-8 by human osteoblasts [43,45]. IL-8 attracts activated macrophages and neutrophils (PMNs), and together with osteoclasts, determines bone loss over time [43]. The statistically significant direct correlation between one-year bone loss and the level of IL-8, in spite of the lack of inflammation, could be explained by this mechanism. However, the highest level of IL-6 and IL-8 was observed in patients 4 and 7 from Group 1 (Table 1). The same patients registered significant peri-implant bone loss at one-year follow-up (Table 4), with the pattern of resorption noticed for these two implants leading to qualify them as unsuccessful, according to Albrektsson's implant success criteria (vertical bone loss less than 0.2 mm/year after the first year post-implantation) [46].

TNF-α, a key biomarker of bone remodeling, was highlighted by several studies as a potential tool for the prognosis and progression of periimplantitis and periodontitis due to its increasing values with disease progression [5,47]. Gomes and co-workers found a significantly higher concentration of TNF-α in individuals who developed periimplantitis after 5 years [5]. In our pilot study, the values of TNF-α in implant patients were similar to those measured in healthy patients, and the biomarker was not correlated to peri-implant bone loss.

The oxidative stress, expressed by shifting the equilibrium between production or activation of free radicals and the antioxidant defenses of the host, in favor of the first one, will potentially lead to the damage of host tissues [15]. Normal saliva contains enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase, peroxidase, catalase, as well as non-enzymatic antioxidants: vitamins C and A, uric acid, albumin and glutathione, among others, with an important role in neutralizing free radicals [14]. The imbalances in the levels of free radicals, reactive oxygen species and antioxidants in saliva may play an important role in the onset and development of periimplantitis. Therefore, total antioxidant activity was determined in the saliva of the subjects enrolled in Group 1 and Group 0 (reference) using the "total antioxidant status (TAS)" kit produced by Randox. The results showed that both groups, implant patients and the healthy controls, included in the study have normal or lowered values of total antioxidant activity in saliva. However, the lowest TAS was noticed in patients 4 and 7 of Group 1. These two patients registered a significant peri-implant marginal bone loss at one year. Additionally, overall, a strong negative correlation was found between marginal bone loss and TAS (Figure 4). This is in accordance with the study published by Novaković and coworkers [14] on total antioxidant activity in the oral cavity before and after periodontal non-surgical treatment. The authors found correlations between clinical periodontal parameters and the level of salivary antioxidants. They demonstrated the importance of TAS assessment in saliva for monitoring of periodontal disease. Continuous secretion and contact with the oral environment reflect a remarkable accuracy in describing all events in the oral cavity, both at the cellular and molecular level [14].

Lactate dehydrogenase (LDH) is an essential enzyme, detectable in normal conditions in the cytoplasm of almost every cell of the human body, being a catalysator of lactate production via pyruvate during anaerobic glycolysis [13]. Its extracellular presence is always related to cell necrosis and tissue breakdown, being a useful biomarker for periodontal disease diagnosis and monitoring [48]. In the present study, the lower levels of LDH in Group 1, implant patients, similar to the mean values measured in Group 2, worked in parallel, reflecting the absence of tissue destruction. No correlation between bone resorption and LDH was noticed.

In spite of the limitations of the present preliminary study, and the lower number of enrolled patients, the strong correlation between marginal bone resorption and total antioxidant status (TAS) in saliva and some proinflammatory cytokines (IL-6 and IL-8) suggests that the application of these simple salivary tests could be associated with the occurrence of complications such as peri-implant bone loss. Low-cost point-of-care devices could be developed to assess patients before dental implants' insertion, and the ones with

low TAS values or increased proinflammatory cytokines levels will be advised to have more regular follow-ups for a close monitoring and prevention of more severe complications.

These markers assessed in the present pilot study were shown to be particularly useful in monitoring peri-implant bone loss, especially IL-6, IL-8 and TAS. However, multicenter studies on larger samples and different implant brands are required for more conclusive results.

5. Conclusions

In the present preliminary study, the total antioxidant activity (TAS) in saliva and the assessment of proinflammatory cytokines (IL-6 and IL-8) were found to be associated with the risk of peri-implant bone loss over time.

Further investigations on the salivary level of proinflammatory cytokines and other important biomarkers, as well as on the salivary antioxidant defense mechanism, need to be performed to provide early diagnosis and efficient monitoring of periimplantitis so that a follow-up program and early treatment can be established for vulnerable patients.

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