CLINICAL, MICROBIOLOGICAL AND INFLAMMATORY EVIDENCE OF THE EFFICACY OF COMBINATION THERAPY INCLUDING SERRATIOPEPTIDASE IN THE TREATMENT OF PERIIMPLANTITIS

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The present study was aimed to evaluate the effect of introducing administration of the proteolytic enzyme serratiopeptidase in the combined mechanical-antibiotic treatment of periimplantitis (PI). Two randomized groups of 64 adults with a diagnosis of PI were studied over a 6-month period. All patients were treated with a combined mechanical and antibiotic protocol for 15 days. The experimental group (EG) was administered antibiotic and serratiopeptidase, while the control group was administered antibiotic alone. To evaluate the effects of the two treatment protocols, clinical and radiographic indices, the concentration of IL-1β, IL-6 and TNF-α in the gingival crevicular fluid, the amount of total bacterial DNA and the presence of specific bacteria were assessed at baseline and at 6 months from treatment. Success rates of combined treatments at 6 months were 96.9% and 78.1% for the EG and CG respectively ($P \leq 0.01$). Implants of the EG showed greater enhancement of clinical, microbiological and inflammatory parameters as compared to those of the CG. Microbiological analyses showed that resistance to combined therapy was constantly associated with the isolation of bacterial species that are not common periodontal pathogens (mainly *S.aureus* and *P.aeruginosa*). The data demonstrate that the addition of serratiopeptidase to combined mechanical-antibiotic treatment protocols of periimplantitis significantly improves outcomes and suggest that serratiopeptidase acts at different levels during the healing process.

The introduction of biomaterials as substitutes for damaged or diseased natural tissues is always associated to an increased risk of the occurrence of infections caused by bacterial biofilms developing at the surface of implanted devices (1). Although the incidence of these infections is generally low, the enormous number of biomaterials that are implanted worldwide annually makes this problem a very relevant one. The situation is made serious by the fact that in several cases mortality rates associated

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with these infections are significant (2). The peculiar physiology of bacterial biofilms makes early diagnosis a quite difficult issue, significantly biasing any option of a simple non-invasive pharmacologic treatment (3). In most cases, once clinically evident, these infections are difficult to treat, and the surgical substitution of implanted biomaterials is often necessary (4). This figure is not substantially different in the case of biomaterials implanted in the oral cavity. In fact, substitution of lost natural teeth by different types of implants is complicated at different rates by implant centered infections, commonly known as peri-implantitis (PI) (5). PI is a process often characterized by severe inflammation affecting peri-implant tissues and causing various degrees of loss of the implant-supporting bone (5) which in most cases, probably begins with colonization of oral bacteria at the implant neck surface. These bacteria, or possibly other opportunistic species, can subsequently colonize the surface of implants, moving apically and causing progressive bone loss. As in other fields, researchers have devoted great effort in the search for protocols able to resolve PI (6-10) but, although there is a general consensus that treatment of PI should include anti infective agents (11) coupled with surgical procedures favoring access for removal of the bacterial biofilm (12), no single method coupled to surgical treatment of peri-implantitis has proved to be superior (6). Moreover, there is limited information on the long-term outcome of treatment of peri-implantitis and on the role of systemic antibiotics as an adjunct to therapy of PI. Many studies were focused in the past on the development of filling materials and protocols able to promote re-osseointegration of bone defects deriving from PI (13-15) while other authors have shown that surgical treatment aiming to create modified, smooth and polished implant surfaces in combination with systemic antibiotics can resolve PI (16). More recently, Albouy at al. (17) showed that resolution of PI with surgical treatment alone is possible but treatment outcome is influenced by characteristics of implant surface.

Previous *in vitro* studies performed by our research group showed that serratiopeptidase, a bacterial protease commercially available as an oral anti-inflammatory drug, and favoring the penetration of antibiotics at infected sites, is able to significantly enhance the activity of several antibiotics against bacterial biofilms (18). Different *in vivo* trials also showed the ability of this enzyme to enhance therapeutic outcomes of antibiotic therapy in the treatment of different prosthetic infections (19-20).

The present study aims to evaluate the possible contribution of combined antibiotic and seratiopeptidase therapy in the treatment of PI.

MATERIALS AND METHODS

Study population

The design was a single masked, randomized 6-month clinical intervention trial, including two study groups with a diagnosis of peri-implantitis. To be included in the study, subjects had: i) at least 20 teeth in their mouth, ii) at least one endosseous dental implant supporting a restoration and showing clinical and radiographic evidence of PI (i.e. radiographic evidence of bone loss ≥3mm), iii) less than 3 more endosseous dental implants. Subjects who were current smokers, who had relevant systemic diseases or who exhibited numerous or severe caries, generalized moderate to severe chronic periodontitis, or significant soft tissue pathology, and subjects who were pregnant or breast feeding were excluded from the study. The nature of the study was explained to all subjects who met the inclusion criteria and they were asked to indicate their acceptance by voluntarily signing an informed consent complying with criteria of the declaration of Helsinki.

Between March 2008 and September 2010, 247 subjects were screened and 128 (mean age 37.4 ±7.0 years, range 21-53 years) who met the above-reported inclusion criteria were enrolled in the study. Of these, 67 (52.3%) were male (mean age 37.1±6.8 years, range 21-53 years) and 61 (47.7%) were female (mean age 37.7±7.3 years, range 23-51 years). The 128 subjects were randomly assigned to the experimental group (EG) (mean age 38.2±7.4 years, range 23-53 years) or the control group (CG) (mean age 36.6±6.6 years, range 21-50 years) each consisting of 64 subjects. The EG consisted of 35 males (mean age 38.5±6.9 years, range 25-53 years) and 29 females (mean age 37.8±8.0 years, range 23-51 years). The CG consisted of 32 males (mean age 35.5±6.5 years, range 21-48 years) and 32 females (mean age 37.6±6.7 years, range 28-50 years). Clinicians involved in the study were not informed of allocation of patients in the two groups and study subjects were instructed not to discuss therapy with the study examiner.

Clinical procedures

In each subject, a dental implant was identified for clinical examination and microbiological sampling which

were performed at baseline and at 6 months. Clinical assessments included: (a) plaque accumulation using the modified Plaque Index (mPlI) (21); and (b) gingival bleeding on probing using the modified sulcus bleeding index (mSBI) (21); (c) periodontal probing depth (PPD) assessed to the nearest 1 mm using a Williams color coded periodontal probe. All parameters were evaluated at the four main aspects of the implant (vestibular, mesial, lingual and distal). In the case of mPlI and mSBI, a mean of the four determinations for each implant was reported, while for PPD the highest value obtained for each implant was reported. All clinical measurements were obtained after removing the supra structure.

Intra-oral standardized radiographs of sites of interest were obtained according to a long-cone paralleling technique and using a holding device for standardization purposes as described previously (22) at baseline and at 6 months. Radiographs were analyzed by one of the study investigators blinded to study assignment, to calculate modifications of the depth of bone lesion (DBL).

Microbiological analyses

Sub-gingival plaque samples were collected from each implant both at baseline and at 6 months, after accurate removal of supragingival plaque, using 2 adjacent sterile endodontic paper points (DiaDent paper points ISO-055). The samples were individually placed in 2.0 ml microcentrifuge tubes containing 0.15 ml of sterile DNAse free TE transport fluid (10 mM Tris-HCL, 1 mM EDTA, pH 7.6) and stored one in dry ice and the other one at room temperature and both within 1 hour of collection. Upon arrival at the laboratory, refrigerated samples were stored at -80°C until processed, while samples transported at room temperature were processed immediately by standard cultural techniques to search for uncommon bacterial species. In order to avoid differences in loss of microbiological information, all the refrigerated samples were stored for the same period of time before being further processed.

The presence of bacterial species that are not common in periodontal samples was evaluated by standard microbiological methods. Samples were appropriately diluted and plated on the following solid media: Columbia agar base (containing 5% defibrinated sheep blood), McConkey agar, Mannitol Salt Agar.

Total bacterial DNA was extracted from microbiological samples using the Nucleospin Genomic DNA purification Kit (Macherey-Nagel GmbH Düren, Germany). Samples were centrifuged at 12,000 x g at 4°C and the bacterial pellet was processed for bacterial DNA extraction. In order to enable complete cell lysis, bacterial pellets were suspended and incubated in 20 mM Tris-HCl; 2 mM EDTA, 1% Triton X-100, pH 8.0 supplemented with 20 mg/ml lysozyme and 0.2 mg/ml lysostaphin for 60 min at 37°C. Proteinase K was then added and samples were incubated for 60 min at 56°C. Following lysis, total DNA was purified according to the manufacturer's instructions. Quantification of total amount of bacteria was performed by real time PCR and was normalized for each site to probing depth and expressed as Log fg DNA/ μL per probing depth mm. Total amounts of bacteria were evaluated using the 16S rRNA gene universal primers 357F and 907R (23, 24) using the Maxima® SYBR Green qPCR Master Mix (Fermentas Life Sciences) according to the manufacturer's instructions. Cycling conditions were as described previously (24) and were undertaken using an Applied Byosystems 7300 Real Time PCR system. Quantitative analysis was performed following construction of a standard curve using the universal primers against a serial dilution of a quantified mix of purified bacterial genomes constructed within the laboratory.

In those cases of PI that proved refractory to combined treatment at 6 months, microbiological samples were also processed to evaluate the presence of common bacterial pathogens by molecular methods as described elsewhere (25, 26). Standard dilutions of quantified genomic DNA were constructed from pure cultures of reference strains of the different bacterial species (*Aggregatibacter actinomycetemcomitans* DSM 8324, *Campylobacter rectus* DSM 3260, *Eubacterium saphenum* CCUG 52676, *Mogibacterium timidum* DSM 3998, *Porphyromonas gingivalis* DSM 20709, *Prevotella intermedia* DSM 20706, *Prevotella tannerae* CCUG 34292, *Slackia exigua* DSM15923, *Tannerella forsythia* CCUG 21028, *Staphylococcus aureus* ATCC 6538, and *Pseudomonas aeruginosa* ATCC 27853) for quantification purposes.

Evaluation of inflammatory mediators

The concentrations of IL-1β (sensitivity 0.057pg/ml), IL-6 (sensitivity 0.039pg/ml) and TNF-α (sensitivity 0.106pg/ml) in the gingival crevicular fluid (GCF) obtained from the studied implants were determined both at baseline and at 6 months by high-sensitivity enzyme linked immunosorbent assays (Quantikine HS, R&D System). GCF was collected from the buccal aspect of each implanted site, after removing the supra structure. The area adjacent to the implant to be sampled was isolated by means of cotton rolls, supragingival plaque was carefully removed with a curette, and the extragingival portion of the implant was gently dried with air. Two Periopaper Strips (Interstate Drug Exchange, Amityville, USA) were inserted at the entrance of the gingival crevice and kept there for 30 s. The strips from each implant were then combined and placed in 1 ml of PBS containing a protease inhibitor cocktail (IBI Scientific, Peosta, USA),

transported to the laboratory in dry ice and then stored at -80°C until processed. Before being processed for cytokine analysis, each vial was vortexed for 3 min and samples were clarified by centrifugation at 20,000 x g for 5 minutes at 4°C. Analyses to determine the concentration of each cytokine were performed twice in triplicate, according to the manufacturer's instructions. Results were calculated using the standard curves created in each assay. Concentrations of the cytokine were expressed as pg/ml, assuming data as indicating the total amount of each cytokine per tested site, without normalization to GCF volume variations.

Treatment protocols

All treatments included the administration of antibiotics; the antibiotic of first choice was amoxicillin-clavulanic acid (2,000 mg/day in 2 administrations), while the antibiotic of second choice was clindamycin (1,200 mg/day in 2 administrations). All patients were prescribed therapy according to the group they were allocated. Patients of the EG were prescribed the antibiotic and serratiopeptidase (2 x 5mg every 12 hours) for 15 days, starting from 24 hours before mechanical treatment. Patients of the CG were prescribed the antibiotic alone for 15 days, starting from 24 hours before mechanical treatment All mechanical treatments were carried out following removal of supra-structures and performing all baseline measurements; supra-structures were remounted at the end of mechanical treatment. All mechanical treatments were performed by the same operator who was unaware of patient assignment to either the EG or CG. After adequate local anesthesia the infected site was exposed by means of a crestal incision and if necessary by intrasulcular incisions of adjacent teeth. Attention was paid to minimize surgical trauma. Granulation tissue was accurately removed and the site was extensively washed with 1.5% hydrogen peroxide and sterile saline. Sutures were applied according to necessity and removed 10 days after intervention.

Statistical analysis

Evaluation of significance of differences in clinical, microbiological and inflammatory parameters among groups of experimental and control sites was performed by Student *t*-tests and by Fisher's exact test, performed at a significance level of $p \leq 0.01$, using statistical analysis tools of the Microsoft Excel software and the online resource available at http://www.quantpsy.org/.

RESULTS

Effect of treatment regimens on clinical parameters

The two groups of PI affected implants that were treated by two different combined treatment regimens one including administration of both antibiotics and serratiopeptidase (EG) and the other antibiotics alone (CG) showed significantly different overall clinical outcomes at 6 months after treatment. In fact only 2 of 64 implants (3.1%) of the EG still showed evident signs of PI at T6, as compared to 14 of 64 (21.9%) of the CG (*P* Fisher ≤ 0.01).

Mean values of the indices studied obtained from both EG and CG before and after combined treatment regimens are reported in Table I. Although both combined treatment regimens resulted in a very significant reduction of all studied indices of disease (see values of *P* for the comparison of values at T0 and at T6 in Table I) values obtained at T6 from the EG were significantly lower than those obtained from the CG, indicating that administration of serratiopaptidase enhanced clinical outcomes of combined therapy. The above-mentioned differences were not only the consequence of the greater incidence of failures in the CG; in fact, if results of successful treatments of the two groups are compared, mean values of both mSBI and DBL at T6 are still significantly lower in the EG than in the CG (Table II), indicating that combined therapy including serratiopeptidase is possibly implicated also in favoring healing. No significant differences were observed at either T0 and T6 that were related to gender (data not shown).

Effects of treatment regimens on microbiological and inflammatory parameters

Treatment regimens used for both the EG and the CG significantly reduced bacterial colonization of the studied implants, as shown by comparison of amounts of bacterial DNA detected at T0 and T6 (data not shown). Moreover, the amounts of bacterial DNA detected at T6 from implants of the EG were significantly lower than those detected from those of the CG ($P \leq 0.01$) (data not shown).

Treatment regimens for both the EG and the CG significantly reduced the flow of GCF at the studied sites and its overall content of inflammatory mediators (IL-1, IL-6 and TNF-α), as evident from comparison of mean values obtained at T0 and T6 (Table III). The flow of GCF and its mean content of inflammatory mediators was significantly lower in the EG compared to the CG (Table III). These

Table I. *Mean values (± standard deviation) of clinical indices obtained at implanted sites of the experimental group (EG) and control group (CG) both before combined treatment (T0) and 6 months after it (T6).*

	Mean (±SD)			*P**
	Overall	EG	CG	EG *vs* CG
mPlI (T0)	2.40 (±0.49)	2.36 (±0.48)	2.44 (±0.5)	0.37
mPlI (T6)	1.14 (±0.48)	1.02 (±0.45)	1,27 (±0.48)	**<0.01**
mSBI (T0)	2.16 (±0.58)	2.16 (±0.6))	2.16 (±0.57)	1
mSBI (T6)	0.90 (±0.60)	0.66 (±0.54)	1.14 (±0.56)	**<0.01**
PPD (T0)	6.42 (±0.92)	6.30 (±0.95)	6.55 (±0.87)	0.12
PPD (T6)	2.87 (±1.32)	2.48 (±0.73)	3.25 (±1.64)	**<0.01**
DBL (T0)	4.27 (±1.08)	4.19 (±1.11)	4.36 (±1.06)	0.37
DBL (T6)	2.70 (±1.22)	2.23 (±0.89)	3.17 (±1.34)	**<0.01**
	*P** (T0)*vs*(T6)			
mPlI	**<0.01**	**<0.01**	**<0.01**	
mSB	**<0.01**	**<0.01**	**<0.01**	
PPD	**<0.01**	**<0.01**	**<0.01**	
DBL	**<0.01**	**<0.01**	**<0.01**	

**Values obtained by performing the Student's t-test on data obtained from the single sites. Values of P indicating significant differences are evidenced in bold.*

Table II. *Mean values (± standard deviation) of clinical indices obtained at implanted sites of the experimental group (EG) and control group (CG) both before combined treatment (T0) and 6 months after it (T6), after exclusion of clinical failures.*

	Mean (±SD)			*P**
	Overall	EG	CG	EG *vs* CG
mPlI (T0)	2.37 (±0.48)	2.37 (±0.49)	2.37 (±0.49)	0.97
mPlI (T6)	1.07 (±0.44)	1.01 (±0.46)	1,14 (±0.41)	0.13
mSBI (T0)	2.05 (±0.54)	2.12 (±0.59))	1.96 (±0.45)	0.1
mSBI (T6)	0.73 (±0.45)	0.61 (±0.49)	0.88 (±0.33)	**<0.01**
PPD (T0)	6.31 (±0.86)	6.29 (±0.93)	6.32 (±0.77)	0.83
PPD (T6)	2.40 (±0.49)	2.39 (±0.49)	2.40 (±0.50)	0.82
DBL (T0)	4.14 (±1.03)	4.18 (±1.09)	4.10 (±0.94)	0.70
DBL (T6)	2.34 (±0.76)	2.16 (±0.75)	2.57 (±0.71)	**<0.01**
	*P** (T0)*vs*(T6)			
mPlI	**<0.01**	**<0.01**	**<0.01**	
mSBI	**<0.01**	**<0.01**	**<0.01**	
PPD	**<0.01**	**<0.01**	**<0.01**	
DBL	**<0.01**	**<0.01**	**<0.01**	

**Values obtained by performing the Student's t-test on data obtained from the single sites. Values of P indicating significant differences are evidenced in bold.*

Table III. *Mean concentrations (± standard deviation) of inflammatory mediators (pg/μl) and mean volumes of GCF (μl) obtained frrom implanted sites of the experimental group (EG) and control group (CG) both before combined treatment (T0) and 6 months after it (T6).*

	Mean (±SD)			*P**
	Overall	EG	CG	EG *vs* CG
IL-1 (T0)	21.2 (±3.1)	21.5 (±3.5)	20.8 (±2.6)	0.22
IL-1 (T6)	8.0 (±2.5)	6.7 (±1.7)	9.25 (±2.6)	**<0.01**
IL-6 (T0)	0.15 (±0.04)	0.16 (±0.03))	0.16 (±0.04)	0.35
IL-6 (T6)	0.08 (±0.04)	0.07 (±0.02)	0.1 (±0.04)	**<0.01**
TNFα (T0)	0.13 (±0.02)	0.13 (±0.02)	0.13 (±0.02)	0.68
TNFα (T6)	0.07 (±0.03)	0.06 (±0.02)	0.09 (±0.03)	**<0.01**
GCF (T0)	0.32 (±0.02)	0.32 (±0.02)	0.32 (±0.02)	0.93
GCF (T6)	0.21 (±0.04)	0.18 (±0.03)	0.24 (±0.04)	**<0.01**
	*P** (T0)*vs*(T6)			
IL-1	**<0.01**	**<0.01**	**<0.01**	
IL-6	**<0.01**	**<0.01**	**<0.01**	
TNFα	**<0.01**	**<0.01**	**<0.01**	
GCF	**<0.01**	**<0.01**	**<0.01**	

**Values obtained by performing the Student's t-test on data obtained from the single sites. Values of P indicating significant differences are evidenced in bold.*

differences were still evident when mean values obtained from successful treatments of the two groups alone were compared (Table IV).

Cultural and molecular characterization of the microbiota collected from implants

Standard cultural techniques were used to search for uncommon bacterial pathogens in samples collected at T0 and T6 from all the studied implants, assuming that if uncommon species were not isolated the infection was caused by common periodontal pathogens. *P.aeruginosa* was isolated at T0 from 12 of 64 (18.75%) implants of the EG and from 13 of 64 (20.3%) implants of the CG; *S.aureus* was isolated at T0 from 13 of 64 (20.3%) implants of the EG and from 11 of 64 (17.2%) implants of the CG. Other uncommon bacterial species were isolated at T0 from 6 of 64 (9.4%) implants of the EG and from 5 of 64 (7.8%) implants of the CG. *P.aeruginosa* and *S.aureus* were isolated contemporarily from the same implant in 2 of 64 (3.1%) cases of the EG and in 3 of 64 (4.7%) cases of the CG. At T6 *P.aeruginosa* and other uncommon Gram negative bacteria were not detected from treatment responsive implants of either the EG or the CG and *S.aureus* was detected from 2 of 62 treatment responsive implants of the EG and from 3 of 50 treatment responsive implants of the CG.

Cultural and molecular techniques were used to characterize the microbial pathogens responsible for the 16 treatment refractory PI (2 in the EG and 14 in the CG). Results of this characterization are reported in Table V as specific DNA over total bacterial DNA extracted from the sample as calculated using the 16SrRNA universal primers. Common periodontal pathogens generally represented a minimal part of the microbiota in these samples and only in 3 of 16 cases (18.75%) (i.e. implants CG7, CG23 and CG 57) they exceeded 1% of the total bacterial DNA. In the case of CG23, *A.actinomycetemcomitans* accounted for 1.19% of the total bacterial DNA isolated from the sample but *S.aureus* was also detected in the sample accounting for 2.36% of the total bacterial DNA. *C.rectus* accounted for 1.23% of the total bacterial

Table IV. *Mean concentrations (± standard deviation) of inflammatory mediators (pg/μl) and mean volumes of GCF (μl) obtained frrom implanted sites of the experimental group (EG) and control group (CG) both before combined treatment (T0) and 6 months after it (T6), after exclusion of clinical failures.*

	Mean (±SD)			*P**
	Overall	EG	CG	EG *vs* CG
IL-1 (T0)	20.7 (±3.0)	21.3 (±3.4)	20.0 (±2.2)	0.22
IL-1 (T6)	7.2 (±1.5)	6.5 (±1.5)	7.98 (±1.3)	**<0.01**
IL-6 (T0)	0.15 (±0.03)	0.16 (±0.03))	0.14 (±0.03)	0.35
IL-6 (T6)	0.07 (±0.02)	0.07 (±0.02)	0.08 (±0.02)	**<0.01**
TNFα (T0)	0.12 (±0.02)	0.13 (±0.02)	0.12 (±0.01)	0.68
TNFα (T6)	0.06 (±0.02)	0.06 (±0.02)	0.07 (±0.01)	**<0.01**
GCF (T0)	0.32 (±0.02)	0.32 (±0.02)	0.31 (±0.02)	0.93
GCF (T6)	0.19 (±0.03)	0.18 (±0.02)	0.22 (±0.01)	**<0.01**
	*P** (T0)*vs*(T6)			
IL-1	**<0.01**	**<0.01**	**<0.01**	
IL-6	**<0.01**	**<0.01**	**<0.01**	
TNFα	**<0.01**	**<0.01**	**<0.01**	
GCF	**<0.01**	**<0.01**	**<0.01**	

**Values obtained by performing the Student's t-test on data obtained from the single sites. Values of P indicating significant differences are evidenced in bold.*

Table V. *Amounts of specific bacterial DNA sequences detected in samples of therapy refractory periimpalntitis cases at T6. Data are expressed as percentage of specific DNA over total bacterial DNA extracted from the sample as calculated using the 16SrRNA universal primers.*

	Patient identification code[a]															
Bacterial species	EG7	EG47	CG7	CG9	CG16	CG23	CG25	CG29	CG32	CG35	CG50	CG53	CG55	CG57	CG62	CG63
A. actinom[b]	ND[c]	0.18	ND	ND	0.11	**1.19**	0.05	ND	ND	ND	ND	ND	0.14	ND	ND	ND
C. rectus	ND	ND	**1.23**	ND	0.25	0.13	O.21	ND	ND	0.09	ND	0.32	ND	0.09	0.11	0.32
E. saphenum	0.21	0.23	0.12	0.38	0.07	ND	ND	0.18	0.23	0.19	0.06	0.46	0.05	0.06	ND	0.07
M. timidum	ND	0.32	ND	0.08	ND	ND	0.34	0.26	0.09	ND	0.14	ND	0.17	ND	0.21	ND
P. gingivalis	0.08	ND	0.45	ND	ND	ND	0.23	ND	0.49	ND	0.21	0.12	ND	**1.44**	ND	ND
P. intermedia	0.43	0.56	0.26	0.11	0.17	0.31	0.07	0.19	0.25	0.36	0.09	ND	0.15	0.24	0.13	ND
P. tannerae	ND	0.21	ND	0.12	0.11	ND	ND	0.07	ND	ND	ND	0.15	0.08	ND	ND	ND
S. exigua	ND	0.16	ND	ND	0.23	ND	ND	0.11	ND	ND	ND	ND	0.24	0.07	ND	ND
T. forsythia	0.17	0.06	0.19	0.14	ND	ND	ND	0.38	0.09	0.12	0.14	0.17	ND	ND	0.06	0.31
P.aeruginosa	ND	**3.98**	ND	ND	**2.43**	ND	ND	**2.67**	**1.46**	ND	ND	**1.78**	**1.93**	ND	ND	ND
S.aureus	**2.39**	**1.56**	ND	**3.76**	0.17	**2.36**	**1.19**	ND	ND	**3.54**	**3.86**	ND	ND	ND	**3.42**	**2.64**
Other species[d]			YES				YES							YES		

[a]*EG: Experimental Group; CG: Control Group;* [b] *Aggregatibacter actinomycetemcomitans;* [c]*ND: not detected* [d] *Bacterial species uncommon in the oral cavity and detected by standard cultural method*

DNA isolated from CG7, but in this case a *Serratia marcescens* strain was isolated from the sample by standard cultural techniques. *P.gingivalis* accounted for 1.44% of the total bacterial DNA isolated from CG57, but in this case a *Klebsiella pneumoniae* strain was isolated from the sample by standard cultural techniques. In 6 of 16 cases (37.5%) *P.aeruginosa* specific DNA sequences were detected and accounted for more than 1% of the sample (2.38±0.9%). In 9 of 16 cases (56.25%) *S.aureus* specific DNA sequences were detected and accounted for more than 1% of the sample (2.49±1.22%). *P.aeruginosa* and *S.aureus* specific sequences were detected contemporary in EG47, although *P.aeruginosa* was more abundant than *S.aureus* (Table V).

DISCUSSION

While the science of biomaterials has significantly improved over the last few decades, offering the concrete possibility to replace damaged tissues by reproducible and reliable surgical techniques, the availability of materials and protocols to prevent and/or block the onset of biomaterial centered infections did not evolve at the same rate. In the case of dental implants, in particular, the problem of PI is a consequence of several concurrent factors (5). In fact, dental implants are intended to work as transmucous devices, in direct contact with the oral cavity and its complex microbiota. Great efforts have been successfully dedicated in the recent past to developing procedures to obtain implant surfaces and designs optimizing the process of osseointegration (27-28). Less attention has been dedicated to improve the characteristics of implant surfaces in direct contact with the gingiva, where early bacterial colonization by oral microbiota and transient bacterial species able to cause PI is most likely to initiate. Susceptibility of the surface of the implant neck to bacterial colonization is important: in fact, greater amounts of plaque accumulated at the gingival margin of an implanted site are more likely to promote gingival inflammation, initiating a periimplant mucositis (11), that is considered by several authors as the initial step of PI. Enhanced levels of pro-inflammatory cytokines essudating from the inflamed marginal tissues are likely to promote evolution of the mucositis to PI by acting both on deeper tissues and on colonizing bacteria. In fact, it is known that elevated levels of certain cytokines (IL1, IL6, TNFα, etc.) may enhance the growth of bacterial biofilms (29), and promote the expression of genes associated with both pathogenicity and virulence (30). The occurrence of PI at a site often affects stability of large prosthetic rehabilitations and implies time, costs and risks. Consequently, much attention has been dedicated in the last decades to search for protocols able to resolve PI (6). At present most protocols adopted to treat PI include the use of anti-infective agents (11) coupled with surgical procedures favoring access for removal of the bacterial biofilm (12). Anti-infective agents commonly used include either disinfectants or antibiotics administered topically or systemically. The existence of a multitude of implants differing from one another in surface and design characteristics make the evaluation of PI treatment protocols extremely difficult and explains why no single protocol of combined treatment of PI has rpvoed to be superior (6), and information on long-term outcomes of different treatments is very limited. A number of protocols that were proposed include mechanical modification of the implant surface to remove the bacterial biofilm and create a smooth implant surface that should be less prone to bacterial colonization (16), however, although these protocols were able to treat PI successfully, no data are available on susceptibility of the modified surfaces to bacterial colonization and on their suitability to re-osseointegration.

In this complex context, basing on previous data obtained by our research group and showing that serratiopeptidase, a bacterial protease commercially available as an anti-inflammatory oral drug, is able to significantly enhance the activity of several antibiotics against bacterial biofilms (18) and to enhance therapeutic outcomes of antibiotic therapy in the treatment of different prosthetic infections (19, 20), we decided to evaluate the effect of introducing serratiopeptidase in a combined protocol to treat PI (based on surgical debridement and administration of systemic antibiotics). The rationale of this combined treatment protocol relies in the additional capacity of serratiopeptidase to make residual bacterial biofilm that survived mechanical debridement of the implant surface, more susceptible to the action

of the antibiotic (18) and in parallel to enhance the penetration of systemically administered antibiotics at the infected site (31).

Overall results obtained in the course of this study confirm that treatment protocols including surgical exposure of implant surfaces followed by their extensive mechanical debridement, associated with systemic antibiotic therapy for two weeks are successful in significantly reducing the clinical signs of PI and the bacterial colonization of implanted sites. The addition of serratiopeptidase to treatment was able to further significantly enhance therapeutic outcomes, improving the success rate from 88.1% of the CG to 96.9% of the EG. Such an enhancement is probably the consequence of mechanisms that are more complex than shown by *in vitro* experiments previously published (18). In fact, when data from successful cases alone were compared, the EG showed a significantly greater improvement of clinical, microbiological and inflammatory parameters compared to the CG. This is possibly the consequence of more efficient eduction of the inflammatory milieu obtained by the administration of serratiopeptidase; in fact, as discussed above, it is known that certain inflammatory cytokines are able to promote the growth of bacterial biofilms (29) and to enhance pathogenicity and virulence in different bacterial species (30), which is essential to promote infection.

It is interesting to note that, in our sample, all microbiological samples obtained from therapy refractory implants contained bacterial species that are not considered typical periodontal pathogens (mainly *S.aureus* and *P.aeruginosa*) that were detected in only a limited number of cases from treatment responsive implants of both the EG and the CG. This observation suggests that, at least in our sample, resistance to combined treatment is mainly a consequence of the presence of atypical bacteria and that better clinical outcomes obtained with the EG are a consequence of enhanced capacity of antibiotic treatment to eradicate bacterial biofilms formed by these bacterial species in the presence of serratiopeptidase.

Overall data presented in this paper demonstrate that the combined systemic administration of antibiotics and serratiopeptidase associated to mechanic debridement of implant surfaces significantly enhances success rates in the treatment of PI without the need to mechanically modify the surface of the implant so altering it original characteristics.

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