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Assessment of periodontal regeneration by analysis of local biomarkers

Gaia Pellegrini[1,2], Giulio Rasperini[1,3], Giorgio Pagni[1,3], William V. Giannobile[4,5], Elena Canciani[1], Federica Musto[1], James Sugai[4], Claudia Dellavia[1]

[1]Department of Biomedical, Surgical and Dental Sciences, Università degli Studi di Milano, Milan, Italy. [2]Research Center for Oral Implantology (CRIO), IRCCS Galeazzi Orthopaedic Institute, Milan, Italy. [3]Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy. [4]Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor USA. [5]Department of Biomedical Engineering, College of Engineering, University of Michigan USA

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SUMMARY

Aim: to assess the expression profile of biomarkers associated with healing of epithelium, connective tissue and bone tissue after two periodontal surgical approaches (regenerative surgery and open flap debridement) that heal following respectively a regenerative and reparative pattern.

Materials and methods: 16 patients underwent to a periodontal regenerative procedure (REG) performed with non resorbable titanium-reinforced membrane alone and 9 patients were treated with open flap debridement (OFD). Gingival crevicular fluid was collected in each experimental site immediately before surgery (BL). Periodontal wound fluid was collected 3-5 days, 1, 2 and 3 weeks after surgery. Clinical measurements (probing depth-PD and clinical attachment level-CAL) and radiographs were taken at baseline and at 6 months.

Results: 2 and 3 weeks after surgery, the expression profile of three main factors of connective tissue activity (vascular endothelial growth factor, matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1) was significantly higher in sites that underwent to regenerative procedure than in those treated with open flap debridement. These findings were significantly correlated to clinical data.

Conclusion: Local wound healing biomarkers are strongly associated with periodontal regeneration over traditional periodontal flap procedures. The use of local biomarkers offers potential for real-time assessment of the periodontal tissue repair process.

INTRODUCTION

In presence of periodontal lesion, surgical procedures may be performed to regenerate infrabony defects or to eliminate the suprabony periodontal pockets. Tissue regeneration is a challenge, operator dependent procedure, that aims to reconstruct well organized periodontal ligament (PDL), cementum and alveolar bone. Several studies assessed clinical improvements after periodontal regenerative procedures. However these data cannot be directly related to the amount of tissue regeneration (cementum, bone and PDL), since today it can be confirmed only by histological analysis (Stavropoulos et al. 2011). Tissue biopsy needed for histological confirm of wound healing is an invasive procedure that destroys the benefits of surgical procedure and is limited by evident ethical problems. For this reason, most of the newly proposed biomaterials and surgical procedures for periodontal regeneration are supported in their efficacy only by clinical data or animal studies. Gingival crevicular fluid (GCF) in healthy tissue and periodontal wound fluid (PWF) in post-operative tissue contains a number of cell-signaling molecules that provide information on cellular and tissue activity, as well as the ongoing reparative or regenerative processes (Cooke et al 2006, Morelli et al. 2011). Analysis of PWF has been performed after surgical procedures that heal differently such as tissue autografts or graft of living cellular construct, and the expression of cell-signaling molecules resulted strongly influenced by healing process (Morelli et al. 2011, Cooke et al. 2006). Thus, this non-invasive procedure may be able to discriminate the healing pattern.

Aim of the present study was to assess the expression profile of biomarkers associated with healing of connective tissue, epithelium and bone tissue after two periodontal surgical approaches (regenerative surgery and open flap debridement) that heal following respectively a regenerative and reparative pattern. Vascular endothelial growth factor (VEGF), tissue inhibitor of metalloproteinase 1 (TIMP-1), matrix metalloproteinase-1 (MMP-1), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), osteoprotegerin (OPG) and bone morphogenetic protein 7 (BMP-7) were analyzed, and in particular were searched factors able to discriminate the different healing process that occurs after these surgical procedures.

MATERIALS AND METHODS

A total of 25 patients were enrolled in this prospective clinical observational study. To meet the inclusion criteria, patients had to be healthy, non-smoking and with good oral hygiene condition: full mouth plaque and bleeding scores (FMPS and FMBS) ≤ 20% at study baseline. 16 patients presented at least 1 infrabony periodontal defect with indication for surgical regenerative procedure (pocket depth > 5mm with infrabony component ≥3mm) (group REG). 9 patients had at least 1 suprabony defect that needed to be treated with open flap debridement (pocket depth >5 mm and infrabony component <3mm) (group OFD). Were excluded from the study, patients with compromised medical history, pregnant or lactating women and smokers. The study received the IRB approval from the University of Milan.

STUDY TIMELINE

After enrollment in REG or OFD group, for each patient were fixed 7 appointments (see figure 1):

- Baseline (BL): collection of clinical measurements, intraoral radiographs of the experimental site, GCF and photographs of the experimental site. Immediately after this phase, the surgery was performed (OFD or REG);
- 3-5 days, 1, 2 and 3 weeks after BL: post-surgical clinical assessments, photographs, collection of PWF, polishing of the treated site;
- 6 weeks after BL: membrane removal;
- 6 months after BL: collection of clinical measurements, photographs, intraoral radiographs of the treated site.

Clinical measurements were taken with a UNC15 periodontal probe (Hu-Friedy Manufacturing Company Inc., Chicago, IL, USA), and included:

- FMPS, FMBS on four sites per tooth of the whole mouth;
- Probing depth (PD), recession (REC) and clinical attachment level (CAL) on four sites of each treated tooth.

Post-surgical assessments included the monitor of wound healing. In particular were recorded membrane exposure (for REG sites), flap necrosis, erythema, bleeding and suppuration.

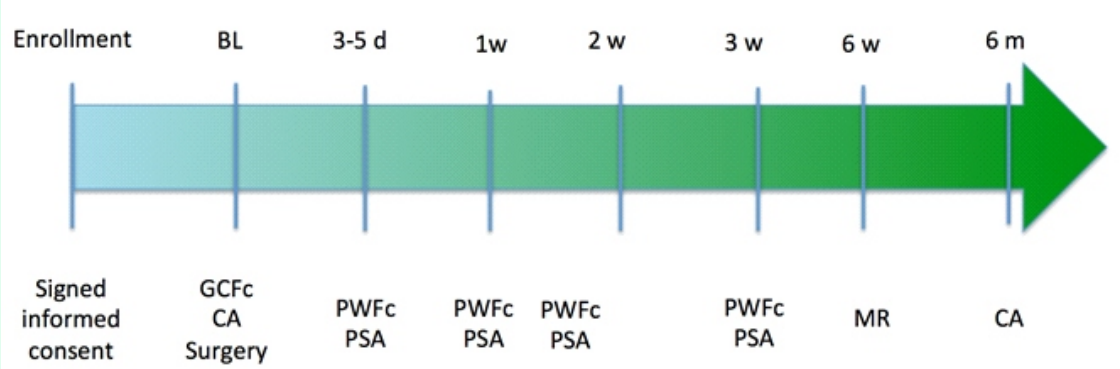

Figure 1: *Timeline of the study. GCFc: gingival crevicular fluid collection; CA: clinical assessments (PD, CAL, FMPS, FMBS, photographs, x-ray); PWFc: periodontal wound fluid collection; PSA: polishing and post-surgical clinical assessments of healing (membrane exposure, necrosis, erythema, bleeding and suppuration of soft tissue); MR: membrane removal; BL: Baseline; d: days; w: weeks; m: months.*

SURGICAL PROCEDURES

After local anesthesia, in sites with infrabony defects a full-thickness flap was elevated with Modified Papilla Preservation Technique (MPPT) (Cortellini et al. 1995) or Simplified Papilla Preservation Technique (SPPT) (Cortellini et al. 1999). In sites with suprabony defects the Modified Widman Flap was performed (Ramfjord and Nissle 1974).

After flap elevation, in all sites (OFD and REG) granulation tissue was removed and defects were cleaned by hand instrumentation and ultrasonic debridement. Infrabony defects (REG) were covered with e-PTFE membranes reinforced in titanium, no biomaterial was used and the only blood clot was let to fill the defect (Figure 2). Otherwise no regenerative procedure was attempted in sites with suprabony defects. REG sites were closed with a single modified internal mattress suture (6/0), thus a tension-free primary closure of the papilla was reached. OFD sites were closed with single external horizontal mattress suture (5/0). Post operative anti-inflammatory regiment was prescribed to all patients: 600mg of ibuprofen immediately before surgery and 6 hours later, subsequent doses were taken only if necessary to reduce pain. A 1 minute rinse with 0.12% chlorhexidine digluconate was prescribed 3 times/day for the first 2 weeks. Sutures were removed at 1 week after surgery and membranes were removed at 6 weeks (Figure 2).

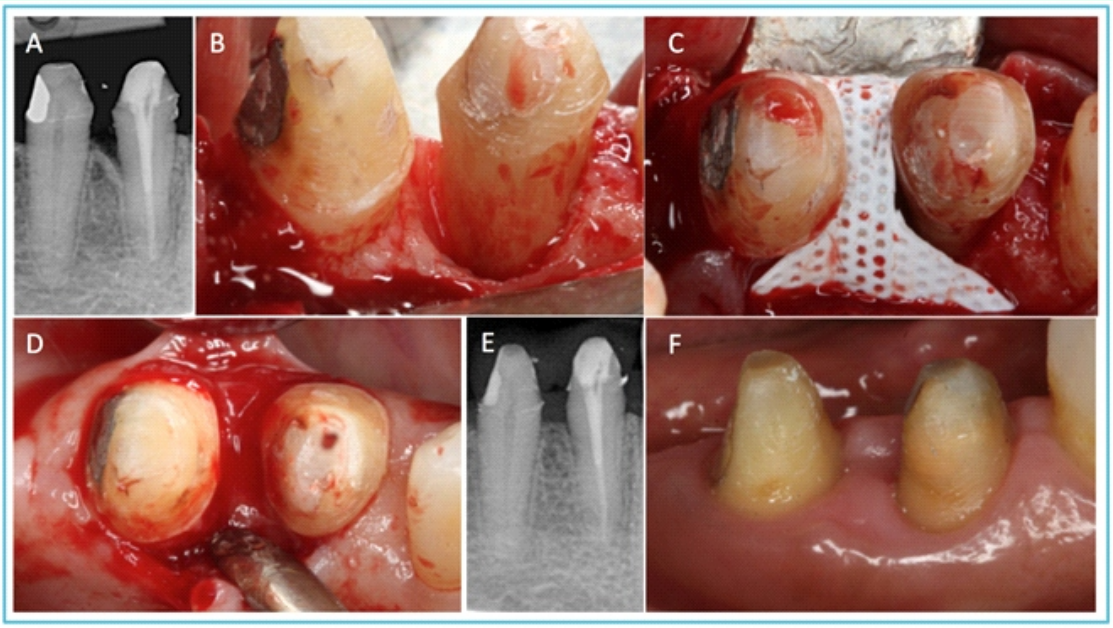

Figure 2: *REG site: A) pre-surgical x-ray; after removal of granulation tissue (B) the infrabony defect was covered with e-PTFE membrane (C); the membrane was removed after 6 weeks (D); x-Rays and photographs were taken 6 months after surgery (E, F).*

GCF AND PWF HARVESTING AND ANALYSIS

GCF was harvested at baseline (immediately before surgery), and PWF was harvested 3-5 days, 1, 2 and 3 weeks after surgery. In each patient, samples were collected from the experimental tooth that underwent regenerative surgery or open flap debridement, and from one healthy control tooth close to the experimental site and that was not interested by surgical procedure. After removing saliva and supra-gingival plaque, a methylcellulose paper strip (Periopaper®, ProFlow Inc., Amityville, NY) was gently inserted in the gingival sulcus or periodontal pocket and was let in for 30 seconds. After that, periostrips were placed into Eppendorf tubes and stored at -20°C until the analysis was performed. Samples were treated for protein extraction as previously reported by Giannobile (Giannobile et al. 1995), and the expression of selected markers (VEGF, TIMP-1, MMP-1, FGF-2, EGF, OPG, BMP-7) was analyzed.

STATISTICAL ANALYSIS

For each group, mean and standard error of each marker were computed at different timepoints. Mean and standard deviation of PD and CAL at baseline and at 6 months of follow-up were calculated. PD reduction and CAL gain were also calculated as the difference between the values taken at baseline and those at 6 months ($p<0.01$, Wilcoxon Mann Whitney signed-rank test for paired data).
To assess differences between groups in the expression of each factor over the time, the Wilcoxon Mann Whitney sum rank test for unpaired data ($p<0.05$) was performed. This test was also used to evaluate differences in PD reduction and CAL gain between REG and OFD groups. Friedman test was used for longitudinal intragroup analysis that evaluated within the same group the variation over the time of the expression of each marker ($p<0.05$). Furthermore, intragroup difference between the expression of factors at each healing timepoint (3-5 days, 1, 2 and 3 weeks) and baseline was also computed by Wilcoxon Mann Whitney signed-rank test ($p<0.05$).
To evaluate if molecular data can be associated to clinical results, Kendall correlation test was used ($p<0.05$). The correlation was computed between values of each marker at 3 weeks and PD reduction or CAL gain.

RESULTS

25 patients (16 REG and 9 OFD) were treated. Demographic data are reported in table 1. No patient was smoker or presented systemic diseases. No differences in values of full mouth plaque and bleeding score were observed at baseline. After surgery no patient reported adverse events, no membrane exposure was observed (for REG group) and all sites healed uneventfully.

	OFD (n=9)	REG (n=16)
Age	58 ± 8	55 ± 9
Gender	4 f; 5 m	12 f; 4 m
FMPS	6.2 ± 4.2	5.5 ± 2
FMBS	3.8 ± 3	3.4 ± 2.5

Table 1: *Demographic data of study population at baseline. n= number; m: male; f: female; FMPS: full mouth plaque score (%); FMBS: full mouth bleeding score (%).*

At the analysis of clinical data resulted that both REG and OFD had a significant reduction of PD and gain of CAL at 6 month after surgery ($p<0.01$). PD reduction and CAL gain in REG sites were higher than in OFD sites ($p<0.01$) (Table 2).

	OFD		REG	
	Baseline	**6 months**	**Baseline**	**6 months**
PD mm	5.56±0.73	2.89± 0.78 §	8.06±1.91	4.13±1.02 §
PD reduction mm	2.67±0.87		3.94±1.29¶	
CAL mm	6.33±2.06	4.44±1.33 §	9.75±2.98	5.38±2.28 §
CAL gain mm	1.89±1.36		4.38±1.02¶	

Table 2: *Mean and standard deviation of PD (probing depth) and CAL (clinical attachment level) at baseline and at 6 months; PD reduction and CAL gain at 6 months for REG and OFD sites.*
¶: significant difference between OFD and REG values, $p<0.01$.
§: significant difference between data at baseline and at 6 months, $p<0.01$.

The expression of each biomarker over the time has been reported in figure 3.

From the analysis between groups, a significantly higher expression of TIMP-1 (at 2 and 3 weeks) ($p<0.05$), MMP-1 (2 weeks $p<0.01$) (3 weeks $p<0.001$) and VEGF (3 weeks $p<0.01$) was observed in REG than in OFD sites. The longitudinal intragroup analysis revealed that after surgery in REG group significant changes occurred in the expression of EGF ($p=0.05$), MMP-1 ($p<0.001$), TIMP-1 ($p<0.001$) and VEGF ($p=0.015$).

Otherwise in OFD group resulted significantly changed only values of MMP-1 ($p=0.012$). The intragroup analysis of difference between the expression of markers at each healing timepoint (3-5 days, 1, 2 and 3 weeks) and baseline showed that:

- in REG sites, TIMP-1 and MMP-1 (both at 3-5 days, 1, 2 and 3 weeks), VEGF (at 3 weeks) and EGF (at 3-5 days, 2 and 3 weeks) were significantly more expressed during healing than at baseline ($p<0.05$);
- in OFD sites, TIMP-1 (at 3-5 days), MMP-1 (at 3-5 days and 1 week) and OPG (at 1 and 2 weeks) were significantly more expressed during healing than at baseline($p<0.05$);

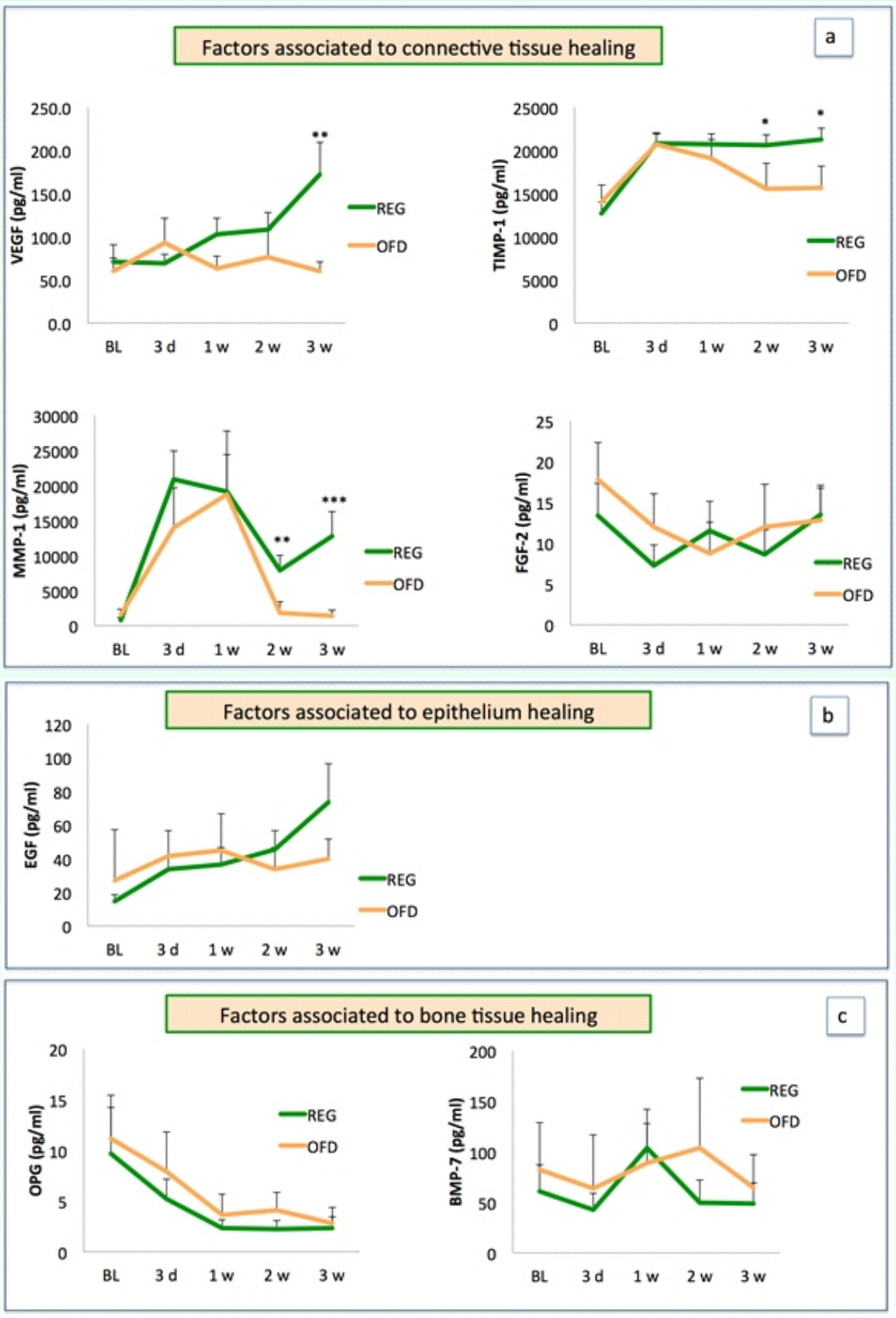

Figure 3: *Mean SE of biomarkers associated to connective tissue (VEGF, TIMP-1, MMP-1, FGF-2) (a), epithelium (EGF) (b), and bone (OPG, BMP-7) (c) healing in gingival crevicular fluid at baseline (BL), and in periodontal wound fluid at 3-5 days, 1, 2 and 3 weeks after surgery in REG and OFD groups. *p<0.05, **p<0.01, ***p<0.001.*

The analysis of correlation between clinical and molecular data showed significant values for (Table 3):

- PD reduction and TIMP-1 (at 3 weeks), ($p<0.05$);
- PD reduction and MMP-1 (at 3 weeks, $p<0.05$);
- CAL gain and VEGF (at 3 weeks, $p<0.005$);
- CAL gain and MMP-1 (at 3 weeks, $p<0.05$).

3 weeks	PD red	CAL gain
VEGF	0.121	0.004*
TIMP-1	0.040*	0.073
MMP-1	0.040*	0.023*
FGF-2	1.304	0.736
EGF	0.397	0.304
OPG	1.297	0.688
BMP-7	0.265	0.354

Table 3: *Correlation between clinical data (PD reduction and CAL gain) and marker expression at 3 weeks. Kendall correlation, *p<0.05.*

CONCLUSION

Data of the present study confirm that open flap debridement of suprabony defects and regeneration of infrabony defects by means of e-PTFE reinforced membrane arise different healing patterns that reflect different clinical results. Both surgical techniques obtained an improvement of clinical outcomes, however sites treated with regenerative procedure had significantly higher pocket depth reduction and clinical attachment level gain than OFD. The current regenerative technique with blood clot stabilization by membrane demonstrated to induce periodontal regeneration in human histological studies (Nyman et al. 1982). It was decided to avoid resorbable biomaterials (membrane or scaffold) since the inflammation that arises during the resorption process may jeopardize the expression of markers observed in this study. Similarly, in order to avoid alteration of the healing by external factors, PWF was harvested at early timepoints (up to 3 weeks) before membrane exposure and removal. In order to compare regenerative and reparative pattern, flap debridement procedure in suprabony defects was chosen since it induces repair healing and the development of long junctional epithelium (Caton & Nyman 1980).

At the analysis of molecular factors, sites undergoing regeneration revealed higher connective tissue activity at 2 and 3 weeks after surgery. In REG sites in this phase of healing (2 and 3 weeks), VEGF, MMP-1 and TIMP-1 were significantly more expressed not only compared to baseline, but also compared to OFD group.

During healing activity, VEGF induces the proliferation and migration of endothelial cells (Barrientos et al. 2014). Together with FGF-2, it stimulates angiogenesis, granulation tissue formation, re-epithelization and tissue remodeling (Morelli et al. 2011, Barrientos et al. 2014). MMP-1 is responsible of collagen type I degradation (Page-McCaw et al. 2007). TIMP-1 is an inhibitor of MMP-1 and promotes anabolic tissue function (cell proliferation and differentiation) (Ries 2014). These two molecules have contrasted activities and their balanced ratio seems to be a prognostic factor for good healing (Muller et al. 2008). The increased expression of TIMP-1 after surgery may suggest the regulation of MMP-1 for a correct healing. These data indicate that in this regenerative model, collagen formation and maturation requires longer periods of time to be completed, possibly exceeding 3 weeks, than in OFD.

Epithelial growth factor (EGF) stimulates re-epithelization and accelerates wound repair with less scaring (Khanbanha et al. 2014). In the present study, expression of this marker was higher at 3 weeks in REG than in OFD group but without significance. This marker may be less sensitive, and a more numerous study population should be analyzed to assess an eventual significance. Bone morphogenetic protein-7 (BMP-7) and osteoprotegerin (OPG) play a role in bone tissue formation, respectively inducing bone mineralization and inhibiting bone resorption. The similar expression pattern of these factors in two groups may indicate that in this early phase of healing, OPG and BMP-7 intervene similarly between two healing patterns. Indeed, expression of BMP-7 in fracture healing has been reported to peak between 14 and 21 days in mouse (Cho et al. 2002), and it has to be considered that this animal has a faster healing activity than human.

From results of the present study, it appears that at 3 weeks after surgery connective tissue activity is significantly more intense than at baseline, as revealed by expression profile of three main factors (VEGF, MMP-1 and TIMP-1). This activity is stronger in sites that underwent to regenerative procedure than in those treated with open flap debridement. These findings are correlated to clinical data: the PD reduction and CAL gain at 6 months resulted significantly related to the expression of connective tissue markers at 3 weeks. It may be claimed that quantitative analysis of connective tissue factors (VEGF, MMP-1 and TIMP-1) performed in PWF at 3 weeks of healing has a predictive value on clinical parameters (amount of PD reduction and CAL gain) that can be evaluated only at 6 months. Furthermore by the assessment of these markers it would be possible to discriminate between two different periodontal healing processes thus limiting invasive histological analysis of block sections.
The major limitation of this study was the limited number of treated patients. It would be interesting to increase the study population and to investigate further factors and timepoints to have more complete and precise information on periodontal reparative and regenerative processes. From a clinical point of view, a non-invasive method to predict and investigate the effects of any procedure on the periodontal complex may be useful.

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