Journal of
PERIODONTAL RESEARCH

J Periodont Res 2017; 52: 388–396

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12403

Local wound healing biomarkers for real-time assessment of periodontal regeneration: pilot study

G. Pellegrini[1,2], G. Rasperini[1,3], G. Pagni[1,3], W. V. Giannobile[4,5], S. Milani[6], F. Musto[1], C. 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, MI, USA, [5]Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, MI, USA and [6]Laboratory G.A. Maccacaro, Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy

Pellegrini G, Rasperini G, Pagni G, Giannobile WV, Milani S, Musto F, Dellavia C. Local wound healing biomarkers for real-time assessment of periodontal regeneration: pilot study. J Periodont Res 2017; 52: 388–396. © 2016 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Background and Objectives: Within the same surgical procedure, a great variability on achievement of clinical outcomes exists and may be associated to different molecular factors related to tissue healing. The aim of the present study was to assess the distribution of clinical success separately in regenerative therapy (REG) and open flap debridement (OFD) to evaluate if factors related with healing of epithelium, connective tissue and bone may be associated to the clinical outcome within each surgical procedure.

Material and Methods: Sixteen patients underwent periodontal REG and nine patients underwent OFD. Periodontal wound fluid was collected at baseline, 3–5, 7, 14 and 21 d after surgery, and expression of wound healing proteins was assessed. Pocket depth and clinical attachment level were taken at baseline and at 6 mo of follow-up. Percentage pocket depth reduction and percentage clinical attachment level gain were computed. Patients were regarded as better or worse responders depending on their percentage pocket depth reduction or percentage clinical attachment level gain.

Results: Higher percentage of better responders was observed in the REG group (68.7%) compared to the OFD group (22.2%). At 21 d, no difference in the profile of most of the proteins emerged, with two exceptions, both regarding REG treatment. Bone morphogenetic protein-7 tended to increase in better responders and to decrease in worse responders. Matrix metalloproteinase-1 increased in worse responders and remained substantially unchanged in better responders.

Conclusion: Local expression of matrix metalloproteinase-1 and bone morphogenetic protein-7 during wound healing is associated with the clinical performance of periodontal regenerative surgery. The use of local biomarkers offers the potential for real-time assessment of the periodontal healing process.

Dr. Gaia Pellegrini, DDS, PhD, Department of Biomedical, Surgical and Dental Sciences, Università degli Studi di Milano, Via Mangiagalli, 31, 20133 Milano, Italy
Tel: +39 (02) 50315405
Fax: +39 (02) 50315387
e-mail: gaiapellegrini.perio@gmail.com

Key words: biomarkers; BMP-7; gingival crevicular fluid; MMP-1

Accepted for publication June 19, 2016

Research on periodontal regenerative therapy (REG) focus on even more minimally invasive surgical approaches (1,2), on biomaterials as scaffolds for blood clot stabilization and cellular migration (3) and on modulation of bioavailability of molecular factors that can move the wound healing towards a regenerative rather than a reparative pattern (4). This last strategy of intervention requires full knowledge of timing and amount of expression of cell signaling proteins that guide the formation of new periodontal ligament after regenerative

surgery. Furthermore, it is important to know the differences in timing and amount of molecule between regeneration and repair so as to intervene selectively on these differentiation factors.

During wound healing, a significant number of cell signaling protein molecules (e.g., growth factors, chemokines or cytokines) and products of cellular activity (enzymes, adhesion molecules) are released in the extracellular matrix subsequent to tissue injury associated with periodontal surgical procedures. At the epithelium level, E-cadherin is an adhesion molecule that plays a key role in maintaining the structural integrity and function of the epithelial barrier (5). Its expression reduces during pocket formation and in periodontal patients compared to healthy patients (6). Epithelial growth factor (EGF) is a molecule displaying an important role on the stimulation of proliferation and differentiation of epithelium and mesenchymal tissues and re-epithelialization of wound after acute injury (7). An increased expression of this protein was found in periodontally diseased patients compared to healthy patients (8). In the connective tissue, transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), matrix metalloproteinase-1 (MMP-1) and tissue inhibitor metalloproteinases (TIMP-1) are cell signaling proteins that work by orchestrating and stimulating angiogenesis, granulation tissue formation, connective tissue regeneration and remodeling (9–11). MMP-1 also plays a role in keratinocyte migration and thus re-epithelialization (12), and in osteoblastic differentiation (13). Bone morphogenetic protein 7 (BMP-7) and osteoprotegerin (OPG) play a role in bone tissue formation, respectively inducing osteoblast differentiation, bone formation/mineralization and inhibiting osteoclastogenesis with the consequent bone resorption (14–16). BMP-7 also demonstrated a significant effect on cementoblasts and resulted in a potent stimulator of cementogenesis *in vivo* (14).

All these molecules are soluble and may be detected within the gingival crevicular fluid in healthy tissues and in periodontal wound fluid (PWF) in postoperative tissues during healing phases. Information about cellular activity, as well as tissue formation, remodeling, destruction and inflammation is provided by the analysis of specific molecules (10,17–19). However, no information is available about whether a particular expression profile of molecules associated to epithelium, connective tissue and bone may be indicative (biomarker) of a clinical outcome after REG and open flap debridement (OFD).

These two surgical procedures are proposed based on the anatomy of periodontal defect with the expectation of different healing patterns (regeneration and repair). However, within the same surgical procedure a great variability on achievement of clinical outcomes exists and may be associated to different molecular factors related to tissue healing.

Aim of the present study was to assess the distribution of clinical success separately in REG and OFD to evaluate if factors related with healing of epithelium (E-cadherin, EGF), connective tissue (TGF-β1, VEGF, FGF-2, MMP-1 and TIMP-1) and bone (BMP-7, OPG) may be associated to the clinical outcome within each surgical procedure.

Material and methods

In this prospective clinical observational study a total of 32 patients were enrolled. Each participant was informed about the study protocol and provided a written Institutional Review Board-approved informed consent form. The study was performed following the principles outlined in the Declaration of Helsinki on experimentation involving human subjects. All procedures and materials in the present study were approved by the ethical committees at the University of Milan (Italy) and at the University of Michigan (USA). Enrolled subjects either had an infrabony periodontal defect needing treatment with the REG group or a horizontal periodontal defect (without infrabony components) needing treatment with OFD (OFD group). Each patient represented the statistical unit and only one defect was treated for each subject. Enrolled patients presented the following inclusion criteria:

- age range: 25–80 years;
- non-smoking (former smokers were included if they had not smoked within 6 mo of the study initiation);
- OFD group: presence of at least one tooth with probing pocket depth > 5 mm and clinical attachment level ≥ 6 mm associated with an intrabony defect ≤ 3 mm;
- REG group: presence of at least one tooth with probing pocket depth > 5 mm and clinical attachment level ≥ 6 mm associated with an intrabony defect of > 3 mm;
- full mouth plaque (FMPS) and bleeding (FMBS) scores 20% at study baseline;
- teeth vital or properly treated with root canal therapy;
- absence of inadequate restorations.

Exclusion criteria were:

- patients chronically treated (i.e., 2 wk or more) with any medication that affect periodontal status (i.e., antibiotics or non-steroidal anti-inflammatory drugs), with clinically significant or unstable organic diseases or compromised healing potential (i.e., connective tissue disorders or bone metabolic diseases);
- pregnant women or lactating;
- patients affected by active infectious diseases, immune-compromised, or taking steroid medications.

Gingival crevicular fluid or periodontal wound fluid harvesting and analysis

In each subject of both groups (REG, OFD), gingival crevicular fluid was collected by an expert operator from the tooth with the target lesion before surgical procedure, and PWF was

collected from the same tooth after surgery. Briefly, before fluid collection, the harvesting site was air-dried and the supragingival plaque was removed by means of a cotton pellet. A methylcellulose paper strip (Periopaper®; ProFlow Inc., Amityville, NY, USA) was inserted into the gingival sulcus, for about 1 mm, until a slight resistance was felt and was left in place for 30 s. All samples were subsequently kept on dry ice and stored at −20°C until needed for analysis as reported by Cooke *et al.* (17). Gingival crevicular fluid was sampled at day 0 (baseline) and PWF was collected 3–5, 7, 14 and 21 d after surgery (for the timeline see Fig. 1).

Before the biomarker analysis, Periopaper® strips (Oraflow, Smithtown, NY) containing gingival crevicular fluid and PWF were thawed at room temperature and proteins were eluted as previously described (20). Biomarker expression was quantified using a Quantibody® custom human slide-based array kit (RayBiotech, Inc., Norcross, GA, USA) for the presence of different biomarkers simultaneously (E-cadherin, EGF, TGF-β1, VEGF, FGF-2, MMP-1, TIMP-1, BMP-7 and OPG) according to the manufacturer's protocol (10). Briefly, each slide contained known concentrations of standards (pg/mL) for each cytokine, used for making serial dilutions to yield a six-point standard curve, with sample diluent serving as the negative control. Standards and experimental samples were incubated overnight at 4°C followed by washing unbound materials. The detection antibody was then bound to the antigens within each well. Cy3 equivalent dye-conjugated streptavidin was pipetted into each well, which bound to the detection antibody associated with immune complexes. The slides were incubated and the fluorescence intensity detected using a laser scanner. The resultant signals of the samples were compared to the standard curve for each of the cytokines to determine the concentrations of each cytokine within the samples. Data were extracted and analyzed using Quantibody Array analysis software (RayBiotech, Inc.).

Clinical and radiographic analysis

Standardized intraoral radiographs of the defect were taken using a Rinn's attachment and a long cone parallel technique at baseline and 6 mo after periodontal surgery.

Intraoral photographs of the experimental sites were taken during surgery, at 1, 2, 3 and 24 wk.

Clinical measurements were taken at baseline and 6 mo after surgery:

- FMPS and FMBS on four sites per tooth of the whole mouth.
- Periodontal parameters on four sites of each tooth treated: probing pocket depth, recession, clinical attachment level (calculated as the sum of the probing pocket depth and recession).

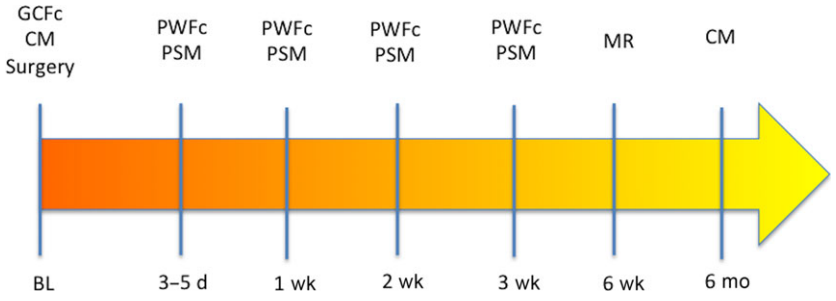

Fig. 1. Timeline of the study. BL, baseline; CM, clinical measurements (probing depth, clinical attachment level, full mouth plaque and full mouth bleeding scores) and intraoral radiographs; d, days; GCFc, gingival crevicular fluid collection; m, months; MR, membrane removal; PSM, post-surgical clinical assessments of healing (membrane exposure, necrosis, erythema, bleeding and suppuration of soft tissue) oral hygiene instruction and polishing performed by means of a rubber cup; PWFc, periodontal wound fluid collection; w, weeks.

All measurements were taken with an UNC periodontal probe (Hu-Friedy Manufacturing Company Inc., Chicago, IL, USA).

Surgical procedures

Immediately before surgery, all patients underwent a careful hygiene phase receiving professional oral hygiene procedures and instructions. After local anesthesia (mepivacaine 2% 1 : 100,000 epinephrine) (Scandonest, Septodont, France) in all sites (OFD and REG) full-thickness flap was incised and elevated. In REG sites the simplified papilla preservation technique (SPPT) or modified papilla preservation technique (MPPT) were adopted (21,22). The SPPF was performed whenever the width of the interdental space was 2 mm or narrower, while the MPPT was applied at interdental sites wider than 2 mm. The intra-sulcular interdental incision (SPPF or MPPT) was extended to the buccal and lingual aspects of the mesial and distal teeth adjacent to the defect. In OFD sites, a modified Widman flap was performed (23).

In both groups, after flap elevation, the granulation tissue was removed and the roots were planed by means of mini-curettes (Gracey; Hu-Friedy) and power-driven instruments (Sonicflex®; Lux, Kavo, Charlotte, NC, USA). Vertical releasing incisions were performed when flap reflection caused tension at the extremities of the flap(s).

Infrabony defects (REG) were covered with a non-resorbable titanium-reinforced completely inert membrane (dense polytetrafluoroethylene, d-PTFE) (Cytoplast®; Osteogenics Biomedical, Lubbock, TX, USA) alone with no bone substitutes, while in horizontal defects regeneration procedures were not attempted (OFD). Buccal and lingual flaps were re-positioned at their original level, without any coronal displacement to avoid any additional tension in the healing area. REG sites were closed for primary intention with a single modified internal mattress suture 5/0 (expand polytetrafluoroethylene, e-PTFE) (Gore-tex®; WL

Gore & Associates, Flagstaff, AZ, USA). OFD sites were closed with single external horizontal mattress suture 4/0 e-PTFE (Gore-tex®; WL Gore & Associates). Vertical releasing incisions were sutured with interrupted sutures.

Postoperative pain and edema were controlled with ibuprofen (600 mg at the beginning of the surgical procedure and 6 h later). Subsequent doses were taken only if necessary to control pain. Patients with ulcers, gastritis and other contraindications to non-steroidal anti-inflammatory drugs received 500 mg acetaminophen. All patients were instructed to apply intermittently an ice bag on the operated area (20 min per hour for 24 h). All patients were instructed to discontinue tooth brushing and avoid trauma at the surgical site for a period of time between 3 and 4 wk. A 60 s rinse with 0.12% chlorhexidine digluconate was prescribed 3 times/d for the first 3–4 wk.

Table 1. Demographic data and basal FMPS, FMBS, PD and CAL (OFD) of subjects under study

	REG (n = 16) Mean (SD)	OFD (n = 9) Mean (SD)
Gender (no. female)	12 (75%)	4 (44%)
Age (years)	55.23 ± 8.74	58.33 ± 7.51
FMPS	5.5 ± 2.0	6.2 ± 4.2
FMBS	3.4 ± 2.5	3.8 ± 3.0
PD (mm)	8.1 ± 1.9	5.6 ± 0.7
CAL (mm)	9.8 ± 3.0	6.3 ± 2.1

CAL, clinical attachment level; FMBS, full mouth bleeding score; FMPS, full mouth plaque score; OFD, open flap debridement; PD, pocket depth; REG, regenerative therapy.

Statistical analysis

The patients participating in this study were grouped by treatment administered (REG or OFD). The clinical outcome was evaluated in terms of percentage pocket depth reduction (PDr%) and percentage clinical attachment level gain (CALg %) in the first 6 mo after surgery, and subjects were classified into two outcome groups: worse responders (below the 50th centile of PDr% and CALg% distribution) and better responders (above the 50th centile).

The between treatments difference was tested with Fisher's exact test.

Protein values were log-transformed (log{value+1}) to reduce the skewness of their distribution. Protein log values recorded at baseline and at 4, 7, 14 and 21 d after surgery were fitted with a linear model for repeated measurements, separately for each treatment group, and possible differences between outcome groups at baseline and in mean daily change

Table 2. Outcome of REG and OFD treatments in terms of PDr% and CALg% (worse responders: < 50th centile, and better responders > 50th centile). Between treatments difference was tested with Fisher's exact test

	REG (n = 16)	OFD (n = 9)	Total	p
PDr%				
< 50th centile	10 (62.5%)	5 (55.6%)	15	1.000
> 50th centile	6 (37.5%)	4 (44.4%)	10	
CALg%				
< 50th centile	5 (31.25%)	7 (77.8%)	12	0.041
> 50th centile	11 (68.75%)	2 (22.2%)	13	

CALg%, percentage clinical attachment level gain; OFD, open flap debridement; PDr%, percentage pocket depth reduction; REG, regenerative therapy.

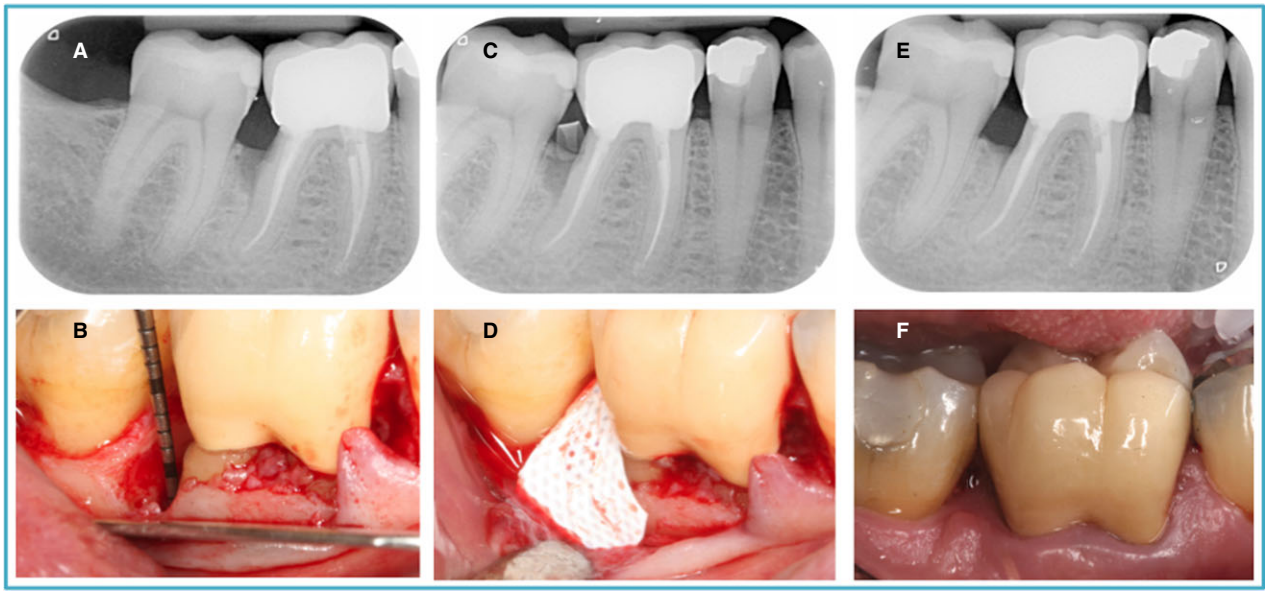

Fig. 2. Regenerative therapy site: (A) pre-surgical X-ray. After degranulation, (B) the infrabony defect was covered with e-PTFE membrane (C, D); X-rays and photographs were taken 6 mo after surgery (E, F).

between baseline and 21 d after surgery were tested. Data were analyzed with SAS PROC MIXED (SAS Institute, Inc. 2008, SAS/STAT® 9.2 User's Guide; SAS Institute, Inc., Cary, NC, USA).

Results

All patients ($n = 16$) of the REG group completed the study. Two patients of the OFD group did not perform the surgery, and five patients of the same group did not show at all control appointments; as their data were incomplete they were excluded from the computation and a total of nine patients of the OFD group were included in the analysis. Samples from 25 patients (16 females and nine males) (nine OFD and 16 REG) were analyzed. Figure S1 shows the patient flowchart. Table 1 reports demographic and clinical data of patients at baseline. No difference in the FMPS and FMBS was found between the two treatment groups. No site in OFD and REG was bleeding on probing at baseline. Uneventful wound healing occurred at all operated sites. At sites treated with REG, no membrane exposure occurred and all membranes were removed at 5–6 wk of healing (Fig. 2).

Table 2 reports the association between outcome and therapy. As for CALg%, the percentage of better responders was higher in the REG group (68.7%) than in the OFD group (22.2%, $p = 0.041$).

Table 3a and 3b report mean protein level (log-scale) at baseline by outcome groups for PDr% and CALg %, respectively. Basal levels of all proteins under study were not significantly different between outcome groups, with the only exception of EGF, which was lower in outcome groups PDr% < 50th ($p = 0.046$) and CALg% ($p = 0.048$), and FGF-2, which was higher in PDr% < 50th ($p = 0.049$), all exceptions regarding the OFD treatment.

Tables 4a and 4b report, separately by outcome groups, the mean daily change in protein level (log-scale) from baseline to day 21 after surgery for PDr% and CALg%, respectively. Mean daily changes of all proteins under study were not significantly different between outcome groups, with the only two exceptions regarding REG treatment. BMP-7 values appeared to increase in subjects with PDr% > 50th centile ($b = +0.074$) and to decrease in subjects with PDr% < 50th centile ($b = -0.072$), the difference between trends being statistically significant ($p = 0.041$) (Fig. 3A). MMP-1 values appeared to increase in subjects with CALg < 50th centile ($b = +0.216$) and to

Table 3. Mean (±standard error) protein concentration (log-scale) at baseline by (a) PDr % and (b) CALg% outcome groups

(a)

		REG		OFD	
Protein	PDr%	Mean ± SE	*p*	Mean ± SE	*p*
E-cadherin	< 50th centile	0.454 ± 0.232	0.899	0.417 ± 0.417	0.407
	> 50th centile	0.504 ± 0.320		0.000 ± 0.000	
EGF	< 50th centile	2.293 ± 0.336	0.976	1.581 ± 0.760	0.046*
	> 50th centile	2.277 ± 0.412		4.121 ± 0.684	
TGF-β1	< 50th centile	1.896 ± 0.809	0.250	1.781 ± 1.124	0.675
	> 50th centile	3.522 ± 1.117		1.128 ± 0.888	
VEGF	< 50th centile	4.054 ± 0.274	0.213	3.830 ± 0.322	0.721
	> 50th centile	3.418 ± 0.435		4.001 ± 0.322	
FGF-2	< 50th centile	1.848 ± 0.533	0.693	3.194 ± 0.163	0.049*
	> 50th centile	1.501 ± 0.673		1.382 ± 0.844	
MMP-1	< 50th centile	4.218 ± 1.084	0.496	4.433 ± 1.446	0.530
	> 50th centile	5.269 ± 0.661		5.781 ± 1.382	
TIMP-1	< 50th centile	9.337 ± 0.160	0.828	9.137 ± 0.296	0.110
	> 50th centile	9.391 ± 0.173		9.781 ± 0.117	
BMP-7	< 50th centile	2.007 ± 0.833	0.738	2.040 ± 1.252	0.787
	> 50th centile	1.556 ± 0.984		1.498 ± 1.498	
OPG	< 50th centile	1.494 ± 0.467	0.815	1.485 ± 0.670	0.553
	> 50th centile	1.322 ± 0.515		2.119 ± 0.772	

(b)

		REG		OFD	
Protein	CALg%	Mean ± SE	*p*	Mean ± SE	*p*
E-cadherin	< 50th centile	0.568 ± 0.348	0.737	0.298 ± 0.298	0.626
	> 50th centile	0.430 ± 0.223		0.000 ± 0.000	
EGF	< 50th centile	2.618 ± 0.294	0.395	2.040 ± 0.630	0.048*
	> 50th centile	2.137 ± 0.340		5.052 ± 0.591	
TGF-β1	< 50th centile	1.034 ± 1.034	0.141	1.383 ± 0.821	0.795
	> 50th centile	3.174 ± 0.793		1.869 ± 1.869	
VEGF	< 50th centile	4.069 ± 0.442	0.497	3.839 ± 3.117	0.596
	> 50th centile	3.700 ± 0.295		4.142 ± 3.378	
FGF-2	< 50th centile	2.464 ± 0.689	0.227	2.773 ± 0.477	0.140
	> 50th centile	1.378 ± 0.486		1.044 ± 1.044	
MMP-1	< 50th centile	2.909 ± 1.612	0.110	5.496 ± 1.215	0.410
	> 50th centile	5.386 ± 0.674		3.408 ± 0.075	
TIMP-1	< 50th centile	9.412 ± 0.234	0.760	9.347 ± 0.246	0.518
	> 50th centile	9.332 ± 0.138		9.685 ± 0.250	
BMP-7	< 50th centile	1.735 ± 1.064	0.915	1.457 ± 0.943	0.516
	> 50th centile	1.884 ± 0.795		2.997 ± 2.997	
OPG	< 50th centile	1.667 ± 0.762	0.653	1.871 ± 0.551	0.715
	> 50th centile	1.322 ± 0.377		1.401 ± 1.401	

BMP-7, bone morphogenetic protein 7; CALg%, percentage clinical attachment level gain; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; MMP-1, matrix metalloproteinase-1; OFD, open flap debridement; OPG; PDr%, percentage pocket depth reduction; REG, regenerative therapy; TGF-β1, transforming growth factor beta 1; TIMP-1, tissue inhibitor metalloproteinase 1; VEGF, vascular endothelial growth factor.
p values refer to the difference in mean basal protein concentration between the outcome groups. *: < 0.05.

Table 4. Mean daily change (± standard error) in protein concentration (log-scale) from baseline to day 21 after surgery by (a) PDr% and (b) CALg% outcome groups

(a)

		REG			OFD		
Protein	PDr%	b ± SE	p_b	p_{diff}	b ± SE	p_b	p_{diff}
E-cadherin	< 50th centile	−0.012 ± 0.011	0.271	0.670	−0.014 ± 0.016	0.416	0.339
	> 50th centile	−0.005 ± 0.014	0.740		0.011 ± 0.018	0.565	
EGF	< 50th centile	0.035 ± 0.028	0.231	0.759	0.043 ± 0.032	0.229	0.222
	> 50th centile	0.049 ± 0.036	0.194		−0.022 ± 0.036	0.555	
TGF- β1	< 50th centile	−0.087 ± 0.037	0.036*	0.989	0.007 ± 0.052	0.889	0.388
	> 50th centile	−0.086 ± 0.048	0.096		0.080 ± 0.059	0.214	
VEGF	< 50th centile	0.044 ± 0.016	0.019*	0.928	−0.008 ± 0.016	0.619	0.801
	> 50th centile	0.042 ± 0.021	0.073		−0.014 ± 0.017	0.441	
FGF-2	< 50th centile	−0.019 ± 0.031	0.547	0.133	0.004 ± 0.028	0.867	0.345
	> 50th centile	0.062 ± 0.041	0.145		−0.037 ± 0.031	0.268	
MMP-1	< 50th centile	0.149 ± 0.040	0.002**	0.125	−0.078 ± 0.043	0.115	0.560
	> 50th centile	0.042 ± 0.051	0.425		−0.038 ± 0.048	0.456	
TIMP-1	< 50th centile	0.019 ± 0.005	0.002**	0.918	−0.015 ± 0.012	0.268	0.592
	> 50th centile	0.018 ± 0.006	0.018*		−0.004 ± 0.014	0.756	
BMP-7	< 50th centile	−0.072 ± 0.039	0.091	0.041*	−0.002 ± 0.060	0.962	0.852
	> 50th centile	0.074 ± 0.051	0.171		0.014 ± 0.067	0.835	
OPG	< 50th centile	−0.013 ± 0.019	0.522	0.136	−0.012 ± 0.037	0.742	0.345
	> 50th centile	−0.064 ± 0.025	0.025*		−0.069 ± 0.041	0.140	

(b)

		REG			OFD		
Protein	CALg%	b ± SE	p_b	p_{diff}	b ± SE	p_b	p_{diff}
E-cadherin	< 50th centile	−0.022 ± 0.015	0.151	0.309	−0.008 ± 0.014	0.597	0.486
	> 50th centile	−0.003 ± 0.010	0.722		0.014 ± 0.027	0.607	
EGF	< 50th centile	0.023 ± 0.039	0.568	0.602	0.029 ± 0.028	0.328	0.274
	> 50th centile	0.048 ± 0.026	0.090		−0.041 ± 0.053	0.458	
TGF- β1	< 50th centile	−0.075 ± 0.053	0.175	0.800	0.029 ± 0.046	0.542	0.655
	> 50th centile	−0.092 ± 0.035	0.021*		0.075 ± 0.087	0.412	
VEGF	< 50th centile	0.047 ± 0.023	0.065	0.843	−0.009 ± 0.013	0.526	0.749
	> 50th centile	0.042 ± 0.016	0.020*		−0.018 ± 0.025	0.487	
FGF-2	< 50th centile	−0.033 ± 0.046	0.483	0.263	−0.016 ± 0.025	0.542	0.860
	> 50th centile	0.031 ± 0.031	0.327		−0.006 ± 0.047	0.895	
MMP-1	< 50th centile	0.216 ± 0.051	0.001**	0.025*	−0.070 ± 0.036	0.099	0.597
	> 50th centile	0.060 ± 0.034	0.101		−0.026 ± 0.069	0.709	
TIMP-1	< 50th centile	0.020 ± 0.007	0.014*	0.740	−0.010 ± 0.011	0.379	0.966
	> 50th centile	0.017 ± 0.005	0.003**		−0.011 ± 0.020	0.599	
BMP-7	< 50th centile	−0.084 ± 0.062	0.196	0.215	−0.014 ± 0.048	0.777	0.431
	> 50th centile	0.013 ± 0.042	0.760		0.071 ± 0.090	0.456	
OPG	< 50th centile	−0.006 ± 0.029	0.821	0.312	−0.039 ± 0.033	0.280	0.926
	> 50th centile	−0.043 ± 0.019	0.043*		−0.032 ± 0.062	0.621	

BMP-7, bone morphogenetic protein 7; CALg%, percentage clinical attachment level gain; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; MMP-1, matrix metalloproteinase-1; OFD, open flap debridement; OPG; PDr%, percentage pocket depth reduction; REG, regenerative therapy; TGF-β1, transforming growth factor beta 1; TIMP-1, tissue inhibitor metalloproteinase 1; VEGF, vascular endothelial growth factor.*p* values refer to mean daily change in each outcome group (p_b) and to the difference in mean daily change between the outcome groups (p_{diff}). *: < 0.05, **: < 0.01.

remain substantially unchanged in subjects with CALg > 50th centile ($b = + 0.060$), the difference between trends being statistically significant ($p = 0.025$) (Fig. 3b). Figure 4A and 4B reports levels of BMP-7 and MMP-1 respectively for PDr% and CALg%.

Discussion

In the present study, the expression of proteins related to epithelium, connective tissue and bone has been observed in PWF during the first 3 wk after REG or OFD, with the aim of detecting possible biological indicators of clinical outcome at 6 mo after surgery.

At baseline, no substantial difference in the level of the evaluated proteins emerged between better and worse responders, for both probing depth and clinical attachment level outcome, and for both treatment groups.

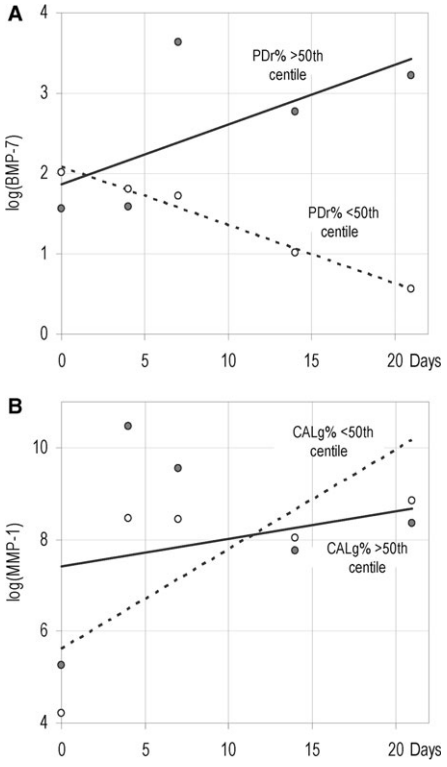

Fig. 3. (A) Time profiles of BMP-7 log values in regenerative therapy treatment by PDr% outcome groups: observed values and fitted lines. (B) Time profiles of MMP-1 log values in regenerative therapy treatment by CALg% outcome groups: observed values and fitted lines. BMP-7, bone morphogenetic protein 7; CALg%, percentage clinical attachment level gain; MMP-1, matrix metalloproteinase; PDr%, percentage pocket depth reduction.

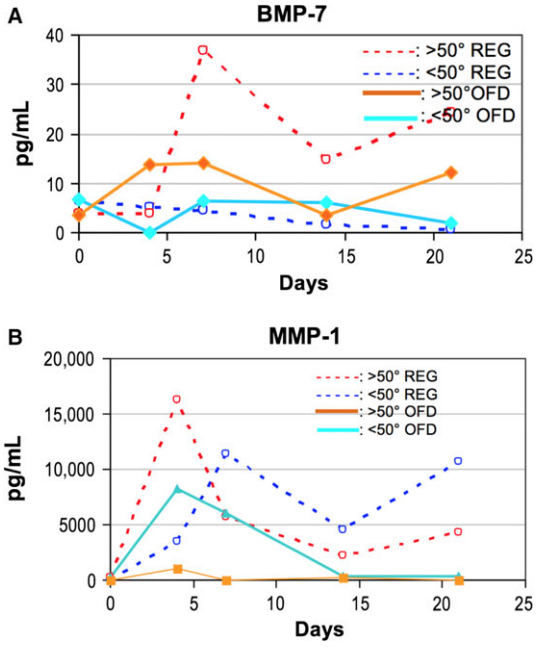

Fig. 4. (A) Time profiles of BMP-7 level in REG and OFD treatments by percentage pocket depth reduction outcome groups. (B) Time profiles of MMP-1 level in REG and OFD treatments by percentage clinical attachment level gain outcome groups. BMP-7, bone morphogenetic protein 7; MMP-1, matrix metalloproteinase; OFD, open flap debridement; REG, regenerative therapy.

In the period of 3 wk after surgery, no important difference in the profile of most of the evaluated proteins emerged, with two noteworthy exceptions (both regarding REG treatment). BMP-7 values tend to increase in better responders and decrease in worse responders. MMP-1 values increase in worse responders and remain substantially unchanged in better responders.

Within the pool of molecules that were considered, MMP-1 and BMP-7 resulted in the most accurate markers to predict the favorable clinical outcome of periodontal regeneration. However, the expression of these proteins was not indicative of the clinical outcome of OFD surgeries. The clinical scenarios (REG and OFD) selected for this study represent two different healing models. It has been histologically demonstrated that the regeneration of periodontal ligament may occur within infrabony defects covered with a space-maintaining barrier (24). In OFD sites, where blood clot is not protected, wound healing occurs through a repair mechanism and formation of a long junctional epithelium (25). It could be hypothesized that MMP-1 and BMP-7 are biomarkers specific for periodontal tissue regeneration, while they do not seem indicators of the reparative process.

MMP-1 is a collagenase responsible for collagen type I degradation and extracellular matrix turnover (11). This protein plays an important role in the pathogenesis of periodontal disease, and reduction of MMP-1 expression has been associated to beneficial effects of periodontal non-surgical therapy (26). During the normal wound healing process, levels of this protein decrease thus opening the proliferative phase and permitting the tissue regeneration (27,28). The predictive role of this metalloproteinase in periodontal tissue regeneration has not been investigated yet in clinical study. Future studies would be designed to evaluate further the role of MMP-1 during the regenerative healing of the periodontal complex and to investigate how the modulation of this protein during

wound healing may favorably modify the outcome of REG. BMP-7 is a growth factor involved in osteogenesis and cementogenesis (14,15). An animal study reported that BMP-7 applied in periodontal defects improves periodontal wound healing (15). A further study reported that expression of BMP-7 in fracture healing peaks between 14 and 21 d in mouse (29).

In the present study, the decrease levels of MMP-1 3 wk after REG in better responders may indicate the transition to the connective tissue regenerative phase of granulation tissue within the chamber under the membrane. The increased expression of BMP-7 may indicate the improved bone, periodontal ligament and cementum formation in better than in worse responders.

A higher percentage of better responders, but only as far as CALg % is concerned, was observed in the REG group (68.7%) compared to the OFD group (22.2%). Similarly, molecular expression pattern resulted in differences between REG and OFD. After REG, healing activity revealed a significant upward trend of EGF, VEGF, MMP-1 and TIMP-1 and a downward trend of TGF-β1 and OPG. Otherwise, in sites treated with the modified Widman flap only OPG was significantly decreased. These data indicate that within the healing space under the membrane that provided a stable chamber for a blood clot, the granulation tissue formation, and connective tissue modeling and maturation lasted for 3 wk and was sustained by these proteins. Otherwise, in OFD sites a coordinated and time-dependent expression pattern of the analyzed molecules was not observed.

When this observational study was planned, neither the expected effect size nor the required size of the study was determined. Nonetheless, the two surgical approaches were found to differ in pocket depth reduction: patients classified as better responders were 78% among those who underwent OFD vs. 31% among those who underwent REG. Under the usual 0.05 risk of type I, the current size of this study has a 0.80 power to detect a 62% difference in the percentage of therapeutic success, and a difference in mean protein concentration at baseline (or in mean daily change), ranging from 1.6 SD (success in PDr% in the REG group) to 2.6 SD (success in CALg% in the OFD group).

Owing to the limited number of subjects under study, the anatomical conformation of the regenerated defects was not compared; for the same reason the comparative analysis of the expression profile of each biomarker in the REG and OFD sites that presented similar clinical behavior (in terms of CALg% and PDr%) could not be performed. Thus, further studies with larger populations and that investigate further factors and timepoints need to be designed. It would be interesting to investigate how defect morphology (remaining bony walls, infrabony component and radiographic angle) and pocket depth at baseline affect protein expression during healing and to have more complete information on molecular activity during periodontal reparative and regenerative processes. Molecules play a fundamental role in the complex evolving scenery of periodontal wound healing and determine the clinical outcome. When the regenerative biomarker profile will be fully established, studies aimed to modulate to the expression profile of biomarkers to guide the tissue regeneration and improve clinical outcomes can be designed more accurately.

Acknowledgements

The Authors thank James Sugai, Dr. Elena Canciani, Dr. Rachele Roncucci and Dr. Ivan Cortinovis for the technical support. This study was founded in part by the University of Michigan Najjar Endowed Professorship to WVG, and in part by University of Milan. The authors declare that they have no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Patient flowchart illustrating patient enrollment, distribution and completion.

References

1. Trombelli L, Farina R, Franceschetti G, Calura G. Single-flap approach with buccal access in periodontal reconstructive procedures. *J Periodontol* 2009;**80**: 353–360.
2. Cortellini P, Tonetti MS. Improved wound stability with a modified minimally invasive surgical technique in the regenerative treatment of isolated interdental intrabony defects. *J Clin Periodontol* 2009;**36**:157–163.
3. Rasperini G, Pilipchuk SP, Flanagan CL *et al*. 3D-printed bioresorbable scaffold for periodontal repair. *J Dent Res* 2015;**94**:153S–157S.
4. Nevins M, Kao RT, McGuire MK *et al*. Platelet-derived growth factor promotes periodontal regeneration in localized osseous defects: 36-month extension results from a randomized, controlled, double-masked clinical trial. *J Periodontol* 2013;**84**:456–464.
5. Fujita T, Hayashida K, Shiba H *et al*. The expressions of claudin-1 and E-cadherin in junctional epithelium. *J Periodontal Res* 2010;**45**:579–582.
6. Nagarakanti S, Ramya S, Babu P, Arun KV, Sudarsan S. Differential expression of E-cadherin and cytokeratin 19 and net proliferative rate of gingival keratinocytes in oral epithelium in periodontal health and disease. *J Periodontol* 2007;**78**:2197–2202.
7. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008;**16**:585–601.
8. Sakai A, Ohshima M, Sugano N, Otsuka K, Ito K. Profiling the cytokines in gingival crevicular fluid using a cytokine antibody array. *J Periodontol* 2006;**77**:856–864.
9. Barrientos S, Brem H, Stojadinovic O, Tomic-Canic M. Clinical application of growth factors and cytokines in wound healing. *Wound Repair Regen* 2014;**22**:569–578.
10. Morelli T, Neiva R, Nevins ML *et al*. Angiogenic biomarkers and healing of living cellular constructs. *J Dent Res* 2011;**90**:456–462.
11. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* 2007;**8**:221–233.
12. Varani J, Perone P, Deming MO *et al*. Impaired keratinocyte function on matrix metalloproteinase-1 (MMP-1) damaged collagen. *Arch Dermatol Res* 2009;**301**:497–506.

13. Hayami T, Kapila YL, Kapila S. MMP-1 (collagenase-1) and MMP-13 (collagenase-3) differentially regulate markers of osteoblastic differentiation in osteogenic cells. *Matrix Biol* 2008;**27**: 682–692.
14. Hakki SS, Foster BL, Nagatomo KJ *et al*. Bone morphogenetic protein-7 enhances cementoblast function in vitro. *J Periodontol* 2010;**81**:1663–1674.
15. Jin QM, Anusaksathien O, Webb SA, Rutherford RB, Giannobile WV. Gene therapy of bone morphogenetic protein for periodontal tissue engineering. *J Periodontol* 2003;**74**:202–213.
16. Jin Q, Cirelli JA, Park CH *et al*. RANKL inhibition through osteoprotegerin blocks bone loss in experimental periodontitis. *J Periodontol* 2007;**78**: 1300–1308.
17. Cooke JW, Sarment DP, Whitesman LA *et al*. Effect of rhPDGF-BB delivery on mediators of periodontal wound repair. *Tissue Eng* 2006;**12**:1441–1450.
18. Kuru L, Griffiths S, Petrie A, Olsen I. Changes in transforming growth factor-beta1 in gingival crevicular fluid following periodontal surgery. *J Clin Periodontol* 2004;**31**:527–533.
19. Kuru L, Kirby AC, Griffiths GS, Petrie A, Olsen I. Changes in soluble adhesion molecules in gingival crevicular fluid following periodontal surgery. *J Periodontol* 2005;**76**:526–533.
20. Giannobile WV, Lynch SE, Denmark RG, Paquette DW, Fiorellini JP, Williams RC. Crevicular fluid osteocalcin and pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP) as markers of rapid bone turnover in periodontitis. A pilot study in beagle dogs. *J Clin Periodontol* 1995;**22**:903–910.
21. Cortellini P, Prato GP, Tonetti MS. The simplified papilla preservation flap. A novel surgical approach for the management of soft tissues in regenerative procedures. *Int J Periodontics Restorative Dent* 1999;**19**:589–599.
22. Cortellini P, Prato GP, Tonetti MS. The modified papilla preservation technique. A new surgical approach for interproximal regenerative procedures. *J Periodontol* 1995;**66**:261–266.
23. Ramfjord SP, Nissle RR. The modified Widman flap. *J Periodontol* 1974;**45**:601–607.
24. Nyman S, Lindhe J, Karring T, Rylander H. New attachment following surgical treatment of human periodontal disease. *J Clin Periodontol* 1982;**9**: 290–296.
25. Caton J, Nyman S. Histometric evaluation of periodontal surgery. I. The modified Widman flap procedure. *J Clin Periodontol* 1980;**7**:212–223.
26. Gonçalves PF, Huang H, McAninley S *et al*. Periodontal treatment reduces matrix metalloproteinase levels in localized aggressive periodontitis. *J Periodontol* 2013;**84**:1801–1808.
27. Tüter G, Kurtiş B, Serdar M. Effects of phase I periodontal treatment on gingival crevicular fluid levels of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1. *J Periodontol* 2002;**73**:487–493.
28. Muller M, Trocme C, Lardy B, Morel F, Halimi S, Benhamou PY. Matrix metalloproteinases and diabetic foot ulcers: the ratio of MMP-1 to TIMP-1 is a predictor of wound healing. *Diabet Med* 2008;**25**:419–426.
29. Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. *J Bone Miner Res* 2002;**17**:513–520.