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Local Wound Healing Biomarkers for Real-Time Assessment of Periodontal Regeneration: pilot study.

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ABSTRACT

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. Aim of the present study was to assess the distribution of clinical success separately in regenerative therapy and open flap debridement in order to evaluate if factors related with healing of epithelium, connective tissue and bone may be associated to the clinical outcome within each surgical procedure.

Materials and Methods: 16 patients underwent periodontal regenerative therapy (REG) and 9 patients underwent open flap debridement (OFD). Periodontal wound fluid was collected at baseline, 3-5, 7, 14 and 21 days after surgery, and expression of wound healing proteins was assessed. Pocket-depth (PD) and clinical-attachment-level (CAL) were taken at baseline and at 6 months of follow-up. Percent-PD-reduction (PDr%) and CAL-gain (CALg%) were computed. Patients were regarded as better or worse responders depending on their PDr% or CALg%. *Results:* Higher percentage of better responders was observed in REG group (68.7%) compared to OFD group (22.2%). At 21 days, no difference in the profile of most of proteins emerged, with two exceptions, both regarding REG treatment. BMP-7 tended to increase in better responders and to decrease in worse responders. MMP-1 increased in worse responders and remained substantially unchanged in better responders. *Conclusion:* Local expression of MMP-1 and BMP-7 during wound healing is associated with the clinical performance of periodontal regenerative

surgery. The use of local biomarkers offers potential for real-time assessment of the periodontal healing process.

INTRODUCTION

Research on periodontal regenerative therapy 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 a 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 epithelium level, E-cadherin is an adhesion molecule that plays a key role in maintaining the structural integrity and function of 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 deseased patients compared to healthy patients (8). In the connective tissue, trasforming growth factor (TGF-b1), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2), 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-epidermization (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 a potent stimulator of cementogenesis in vivo (14).

All these molecules are soluble and may be detected within the gingival crevicular fluid (GCF) in healthy tissues and in periodontal wound fluid (PWF) in post-operative 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 regenerative periodontal therapy and open flap debridement.

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 regenerative therapy and open flap debridement in order to evaluate if factors related with healing of epithelium (E-cadherin, EGF), connective tissue (TGF-b1, VEGF, FGF-2, MMP-1 and TIMP-1) and bone (BMP-7, OPG) may be associated to the clinical outcome within each surgical procedure.

Materials 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 IRB-approved Informed Consent Form. The study was performed following the principles outlined of the Declaration of Helsinki on experimentation involving human subjects. All procedures and materials in the present study were approved by the ethical

committees at University of Milan (Italy) and at University of Michigan (USA). Enrolled subjects either had an infrabony periodontal defect requiring to be treated with regenerative therapy (REG group) or a horizontal periodontal defect (without infrabony components) required to be treated with open flap debridement (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;
- non-smoking (former smokers were included if they had not smoked within 6 months of the study initiation);
- OFD group: Presence of at least one tooth with probing pocket depth (PD) ≥ 5 mm and clinical attachment level (CAL) ≥ 6 mm associated with an intrabony defect ≤ 3 mm;
- REG group: Presence of at least one tooth with PD$\geq$5 mm and CAL ≥ 6 mm associated with an intrabony defect of > 3 mm;
- full mouth plaque (FMPS) and bleeding (FMBS) scores $\leq 20\%$ at study baseline;
- teeth vital or properly treated with root canal therapy;
- absence of inadequate restorations.

Exclusion criteria were:

-patients chronically treated (i.e., two weeks or more) with any medication that affect periodontal status (i.e., antibiotics or NSAIDS), 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 (GCF) or Periodontal Wound Fluid (PWF) Harvesting and Analysis

In each subject of both groups (REG, OFD), GCF was collected by an expert operator from the tooth with the target lesion prior to 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 seconds. All samples were subsequently kept on dry ice and stored at -20°C until needed for analysis as reported by Cooke et al. (2006) (17). GCF was sampled at day 0 (baseline), and PWF was collected 3-5, 7, 14, and 21 days after surgery (for the timeline see Fig. 1).

Prior to biomarker analysis, Periopaper® strips (Oraflow, Smithtown, NY) containing GCF and PWF were thawed at RT 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) for the presence of different biomarkers simultaneously (E-cadherin, EGF, TGF-b1, 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 in order to determine the concentrations of each cytokine within the samples. Data were extracted and analyzed using Quantibody array analysis software.

Clinical and Radiographical Analysis

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

Intraoral photographs of the experimental sites were taken during surgery, at 1, 2, 3

and 24 weeks.

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

- FMPS and FMBS on four sites per tooth of the whole mouth.
- Periodontal parameters on four sites of each tooth treated: PD, recession, CAL (calculated as the sum of PD and recession).

All measurements were taken with a 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, 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, Chicago, IL) and power-driven instruments (Sonicflex® Lux, Kavo, Charlotte, NC). 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, Texas, 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, Flagstaff AZ, USA). Vertical releasing incisions were sutured with interrupted sutures.

Post-operative pain and edema were controlled with ibuprofen (600 mg at the beginning of the surgical procedure and 6 hours later). Subsequent doses were taken only if necessary to control pain. Patients with ulcers, gastritis, and other contraindications to NSAIDs received 500 mg acetaminophen. All patients were instructed to intermittently apply an ice bag on the operated area (20 minutes per hour for 24 hours). All patients were instructed to discontinue tooth brushing and avoid trauma at the surgical site for a period of time ranging between 3 and 4 weeks. A 60 second rinse with 0.12% chlorhexidine digluconate was prescribed 3 times/day for the first 3 to 4 weeks.

Statistical analysis

The patients participating in this study were grouped by treatment administered (REG or OFD). The clinical outcome was evaluated in terms of percent PD reduction (PDr%) and percent CAL gain (CALg%) in the first 6 months 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 days 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 between baseline and 21 days after surgery were tested. Data were analyzed with SAS PROC MIXED (SAS Institute, Inc. 2008. SAS/STATVR 9.2 User's guide. Cary, NC: SAS Institute, Inc).

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 9 patients of the OFD group were included in the

analysis. Samples from 25 patients (16 females and 9 males) (9 OFD and 16 REG) were analyzed. Supporting file 1 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 in all operated sites. In sites treated with regenerative procedure, no membrane exposure occurred and all membranes were removed at 5-6 weeks of healing (Fig. 2).

Table 2 reports the association between outcome and therapy. As for CALg%, the percentage of better responders was higher in REG group (68.7%) than in 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 3c and 3d 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 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 4 a,b 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 periodontal wound fluid during the first three weeks after regenerative therapy (REG) or open flap debridement (OFD), with the

aim of detecting possible biological indicators of clinical outcome at 6 months after surgery.

At baseline, no substantial difference in the level of the evaluated proteins emerged between better and worse responders, for both PD and CAL outcome, and for both treatment-groups.

In the period of 3 weeks 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 the most accurate markers to predict the favorable clinical outcome of periodontal regeneration. However the expression of these proteins wasn't indicative of the clinical outcome of open flap debridement 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 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 further evaluate 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 regenerative procedure. 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 days in mouse (29).

In the present study, the decrease levels of MMP-1 3 weeks after regenerative therapy 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 REG group (68.7%) compared to OFD group (22.2%). Similarly, molecular expression pattern resulted different between REG and OFD. After regenerative therapy, healing activity revealed a significant upward trend of EGF, VEGF, MMP-1 and TIMP-1 and downward trend of TGF-b1 and OPG. Otherwise in sites treated with Modified Widman Flap only OPG resulted significantly decreased. These data indicate that within the healing space under the membrane that provided a stable chamber for blood clot, the granulation tissue formation, and connective tissue modeling and maturation lasted for 3 weeks 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 were 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 REG group) to 2.6 SD (success in CALg% in OFD group).

Due 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 expression profile of each biomarker in REG and OFD sites that presented similar clinical behavior (in terms of CALg% and PDr%) could not be performed.

Thus further studies with larger population and that investigate further factors and timepoints need to be designed. It would be interesting to investigate how defect morphology (remaining bony walls, the infrabony component and the 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 more accurately designed.

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Supporting material

Supporting file 1: Patient flowchart illustrating patient enrollment, distribution and completion.

Figure legend

Fig. 1: Timeline of the study. GCFc: gingival crevicular fluid collection
CM: clinical measurements (PD, CAL, FMPS, FMBS) and intraoral radiographs
PWFc: periodontal wound fluid collection
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.
MR: membrane removal. BL: Baseline; d: days; w: weeks; m: months.

Fig. 2: REG 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 months after surgery (E, F).

Fig. 3a: Time-profiles of BMP-7 log-values in REG treatment by PDr% outcome groups: observed values and fitted lines.

Fig. 3b: Time-profiles of MMP-1 log-values in REG treatment by CALg% outcome-groups: observed values and fitted lines.

Fig. 4a: Time-profiles of BMP-7 level in REG and OFD treatments by PDr% outcome groups.

Fig. 4b: Time-profiles of MMP-1 level in REG and OFD treatments by CALg% outcome groups.

Tables

	REG (n=16) Mean (SD)	OFD (n=9) Mean (SD)
Gender (n. 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

Table 1: Demographic data and basal full mouth plaque score (FMPS), full mouth bleeding score (FMBS), pocket depth (PD), and clinical attachment level (OFD) of subjects under study.

	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	

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		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-b1	<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	

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

p values refer to the difference in mean basal protein concentration between the outcome- groups. *: <0.05.

		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-b1	<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	

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

p values refer to the difference in mean basal protein concentration between the outcome- groups. *: <0.05.

		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-b1	<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

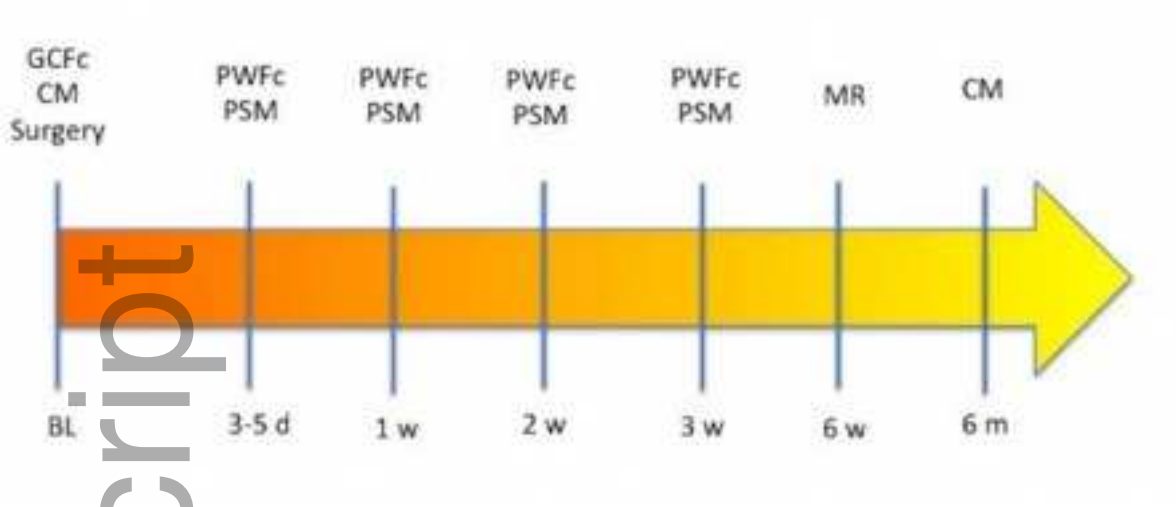
	>50th centile	0.074±0.051	0.171		0.014±0.067	0.835	
OPG	<50th centile	-0.013±0.019	0.522	0.136	-0.012±0.037	0.742	0.345
	>50th centile	-0.064±0.025	0.025*		-0.069±0.041	0.140	

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

		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-b1	<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	-0.033±0.046	0.483	0.263	-0.016±0.025	0.542	0.860

	centile					
	>50th		0.327		0.895	
	centile	0.031±0.031		-0.006±0.047		
	<50th		0.001**		0.099	
MMP-1	centile	0.216±0.051	0.025*	-0.070±0.036		0.597
	>50th		0.101		0.709	
	centile	0.060±0.034		-0.026±0.069		
	<50th		0.014*		0.379	
TIMP-1	centile	0.020±0.007	0.740	-0.010±0.011		0.966
	>50th		0.003**		0.599	
	centile	0.017±0.005		-0.011±0.020		
	<50th		0.196		0.777	
BMP-7	centile	-0.084±0.062	0.215	-0.014±0.048		0.431
	>50th		0.760		0.456	
	centile	0.013±0.042		0.071±0.090		
	<50th		0.821		0.280	
OPG	centile	-0.006±0.029	0.312	-0.039±0.033		0.926
	>50th		0.043*		0.621	
	centile	-0.043±0.019		-0.032±0.062		

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



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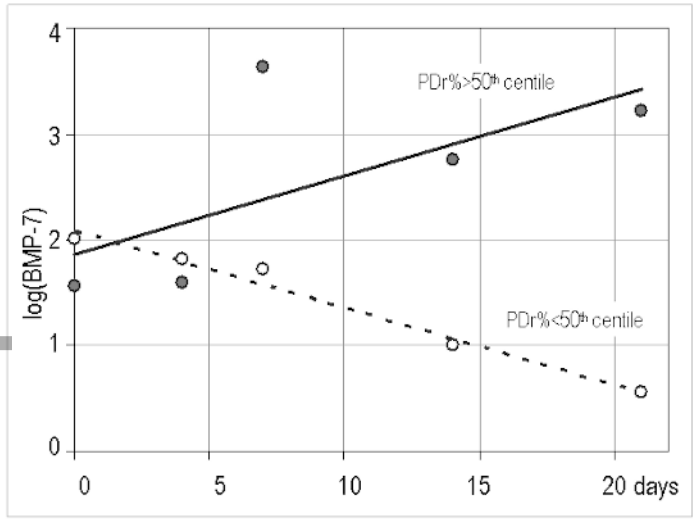

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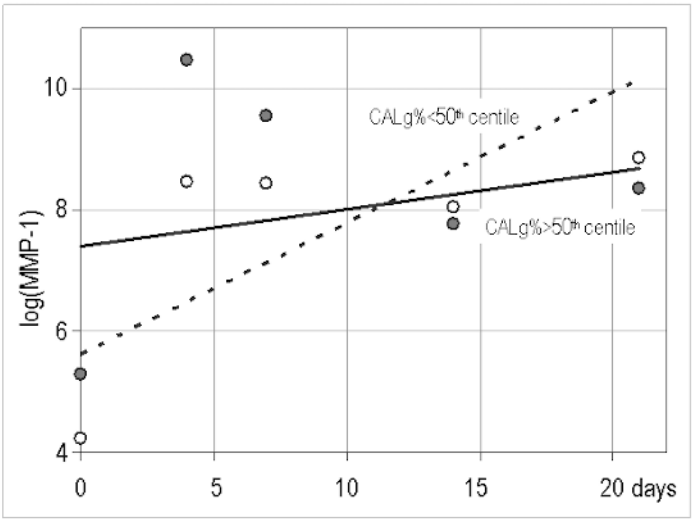

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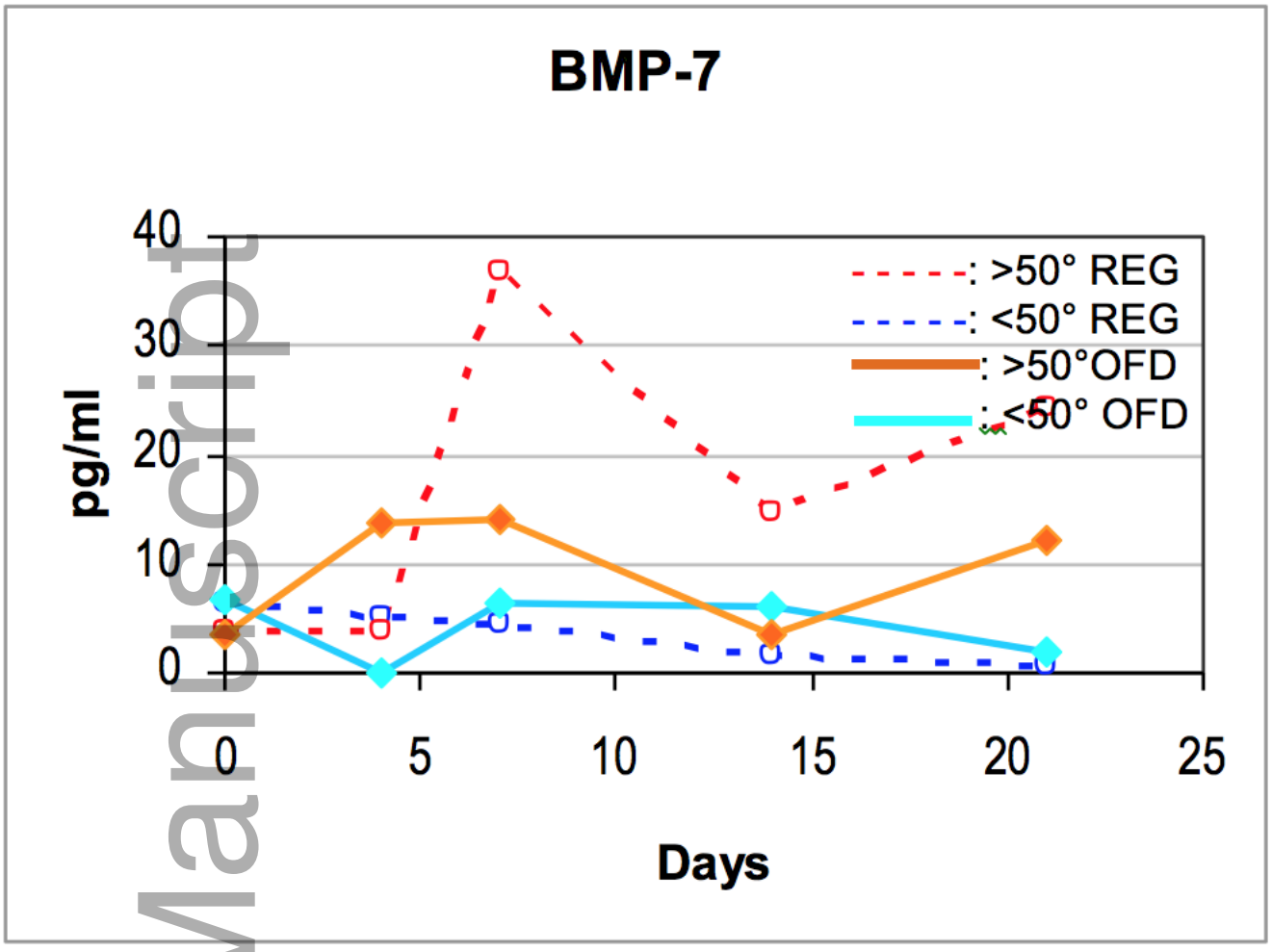

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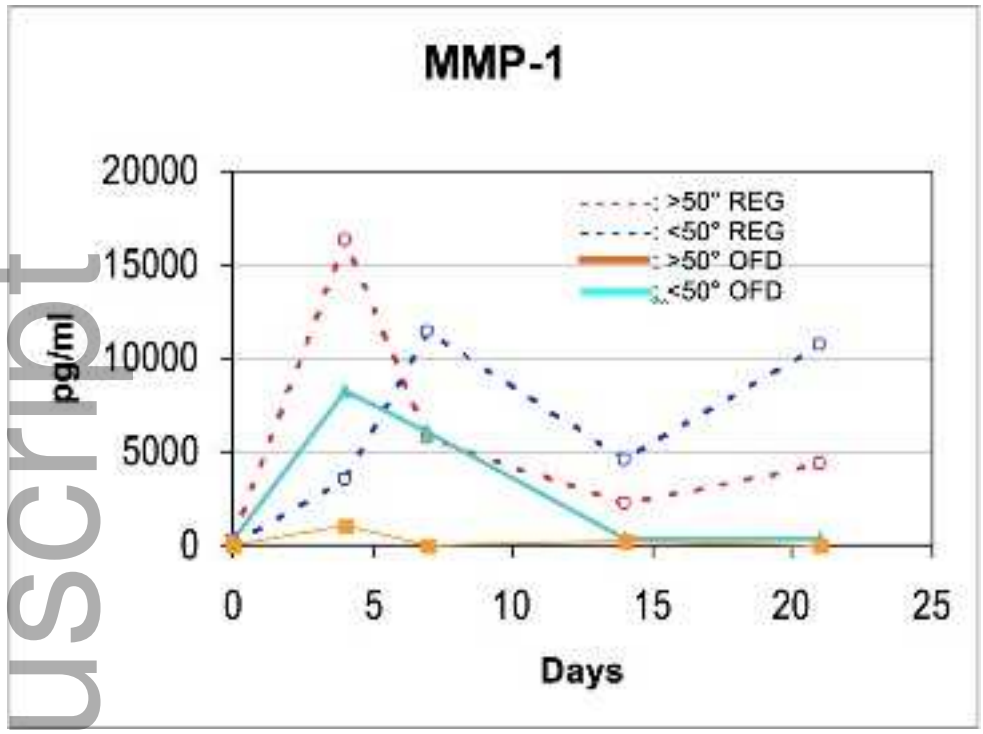

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