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# UNIVERSITÀ DEGLI STUDI DI TORINO

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**Increased early inflammatory response and osteoclastic activity in gingival tissues following conventional osseous resective surgery compared to fibre retention technique: a pilot study.**

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*Running title:* Host-response to resective bone surgery

*Keywords:* bone loss/periodontal; bone resorption; inflammation mediators; periodontitis/surgery.

## **ABSTRACT**

*Background and Objective:* The retention of suprabony connective fibres attached into the root cementum during fibre retention osseous resective surgery (FibReORS) results in a more conservative intrasurgical bony removal, and limits further bone loss and patient morbidity during healing, compared with conventional osseous resective surgery (ORS). This may be a result of the protective effect of preserved connective tissue over the interproximal sites and the less activation of the inflammatory mechanisms. Thus, the aim of this pilot study was to compare the expression of inflammatory and osteoclastic activity markers in gingival tissues following FibReORS and ORS in the early postsurgical phase.

*Material and Methods:* Twenty-six posterior sextants requiring osseous resective surgery were selected in 13 chronic periodontitis patients: 13 sextants were randomly assigned to ORS and 13 to FibReORS in a split-mouth design. Gingival biopsies were collected during the surgical sessions and at suture removal. Tissue samples were analysed to evaluate the expression of proinflammatory and immunity regulatory mediators (interleukin-1 $\alpha$ , C-X-C motif chemokine ligand 5, interferon- $\gamma$  and tumour necrosis factor- $\alpha$ ), cluster of differentiation 14 (CD14; a monocyte/macrophage marker) and TRAP (an osteoclast marker) using immunohistochemical, immunofluorescence and cytofluorimetric analyses, respectively.

*Results:* Postsurgery, a higher number of inflammatory cells and stronger expression of proinflammatory cytokines were observed in the epithelium and connective tissue of ORS gingival samples compared with FibReORS gingival samples ( $p < 0.001$ ). This was accompanied by increased number of CD14-positive and TRAP-cell positivity.

*Conclusion:* Retention of the supracrestal connective fibres appears to reduce the postsurgical intensity of the host-mediated inflammatory response.

## INTRODUCTION

Osseous resective surgery (ORS) is a well-documented treatment approach to eliminate residual periodontal pockets associated with shallow intrabony defects at posterior teeth (1,2). This treatment modality combines the use of both osteoplasty and ostectomy to re-establish physiological bone tissue architecture around teeth in a more apical position (3). This implies the reshaping of positive bone morphology by changing the previous bottom of the defect into the most coronal part of the new interproximal bone surface (4).

Recently, Carnevale proposed a novel approach in resective bone surgery in which the classical method of osseous resection was combined with retention of the supracrestal connective fibres inserted into the root cementum (5). These periodontal fibers, that are always present for approximately 1-2 mm coronally in relation to the bottom of the defect, are removed by the conventional ORS technique. However, ORS with gingival fibre retention (FibReORS) considers the attached supracrestal connective fibres as the bottom of the defect (6,7). This reference point, being more coronal than the real base of the bony defect, minimizes bone resection to the level of buccal/lingual bone adjacent to the interproximal-retained fibres (5). Previous 12-month clinical investigations demonstrated that FibReORS yielded similar probing-depth reduction, but with less clinical attachment loss and bone remodeling, and lower postoperative patient discomfort during the healing process, than ORS (7,8). This may be attributable to the protective effect of preserved connective tissue over the interproximal sites and to the weaker inflammatory host response.

It is widely demonstrated that injury to soft and bony tissues mobilizes a complex sequence of cellular and molecular events that attempts to restore biological form and function. This multistage process incorporates a well orchestrated series of tightly regulated temporal and spatial events involving multiple biological activities, including mobilization of cellular components and expression of factors linked to inflammation and osteogenesis (9). Specifically, mediators such as interleukin-1 $\alpha$  (IL-1 $\alpha$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), C-X-

C motif chemokine ligand (CXCL5) and interferon- $\gamma$  (IFN- $\gamma$ ) play a critical role in the early inflammatory and immune response (10,11). IL-1 $\alpha$  is produced by activated macrophages, neutrophils, epithelial and endothelial cells and it is crucial in periodontal tissue destruction (12). It inhibits osteoblast activity, promotes osteoclast activity and stimulates the production of matrix metalloproteinases (13-15). IL-1  $\alpha$  is part of the pathway of activation of TNF- $\alpha$ , a protein produced mainly by macrophages and a member of a group of cytokines stimulating the acute-phase reaction (16,17).

CXCL5 [also known as epithelial-derived neutrophil-activating peptide 78 (ENA-78)] is a chemokine belonging to the CXC family. It recruits and activates neutrophils and is mainly produced by monocytes, epithelial cells and platelets following stimulation with the primary mediators IL-1 and TNF- $\alpha$  during the acute inflammatory process (11,18).

IFN- $\gamma$  is an important activator of macrophages and exerts immunostimulatory and immunomodulatory effects (19). IFN- $\gamma$  is produced predominantly by natural killer and natural killer T-cells in the innate immune response, and by T-helper 1 and cytotoxic T-lymphocytes in the acquired immune response (20).

Beside the central role of IL-1, TNF- $\alpha$  and IFN- $\gamma$  in regulating osteoclast-mediated bone turnover, the differentiation of mononuclear cells into osteoclasts is a key process in bone loss associated with inflammatory conditions (21-23). Cluster of differentiation 14 (CD 14) and TRAP are markers of mononuclear phagocytes and mature osteoclast activation, respectively (24-26). As far as we are aware, no data are available comparing the early inflammatory response and osteoclast activation following ORS and FibReORS procedures. Therefore, this pilot study was designed to assess the expression of inflammatory and osteoclast activity markers in gingival tissues following ORS and FibReORS to gain further insights into the biological mechanisms involved in the postsurgical inflammatory healing process.

## **MATERIAL AND METHODS**

### ***Study design***

Thirteen healthy patients (three men and 10 women; mean age  $48.4 \pm 4.7$  years), with advanced chronic periodontitis based on the current classification of the American Academy of Periodontology (27), were consecutively selected among individuals undergoing nonsurgical periodontal treatment at the Section of Periodontology, C.I.R. Dental School, Department of Surgical Sciences, University of Turin (Italy), in the period between 1 June and 20 December 2011. Participants received detailed information and signed informed consent. Patients selection, examination procedures and approval by the local Ethical Committee were as previously reported (8).

In brief, all patients completed the aetiological periodontal treatment consisting of motivation and instructions in proper self-performed oral hygiene measures, and full-mouth scaling and root planing. After a healing period of 12 weeks, clinical evaluation was repeated, and patients who presented two contralateral posterior sextants with natural teeth containing at least one defect with residual probing depth  $\geq 5$  mm, persisting bleeding on probing and an associated radiographic intrabony component of  $\leq 3$  mm were selected to participate in the study. Other requirements were a full-mouth plaque score and a full-mouth bleeding score of  $\leq 15\%$  at the time of surgery. Criteria for exclusion from the study were as follows: use of medications affecting periodontal status; contraindications to the periodontal surgery; cigarettes smoking; and pregnancy or breastfeeding for women. Teeth with mobility of Grade II or higher and/or furcation involvement of Grade II or higher were also excluded.

In each patient, two different osseous resective surgical procedures were performed by the same experienced clinician (M.A.) according to a split-mouth design. Shallow intrabony defects were treated using the apically positioned flap approach plus ORS (3) in one randomly selected sextant, whereas in the contralateral segment using the apically positioned flap approach plus FibReORS (6). Overall, 26 posterior sextants were treated: 13 sextants with ORS and 13 sextants with FibReORS. Details regarding surgical procedures were as described previously (8).

### ***Collection of gingival biopsies***

During the surgical session (baseline;  $T_0$ ) gingival biopsies were harvested from ORS- and FibReORS-treated sites. Tissue specimens were collected from the two interdental sites with the greatest probing-depth values in the sextant at the time of presurgical recordings and were randomized using computer-generated randomization table (1:1) to receive histological/molecular analysis or cytofluorimetric evaluation. Ten days after surgery ( $T_1$ ) sutures were removed, and tissue biopsies were repeated on the same sampling sites with a microsurgical blade (USM 6700; Sable industries, Kitchener, Canada). Upon excision, tissue specimens were placed in physiological solution and transported, within 24 h, to the Department of Experimental Medicine, Tissue Engineering and Regenerative Medicine Laboratory, Second University of Naples (Italy) for histological and molecular analyses. The biopsy was performed by the clinician charged of the surgical therapy (M.A.), while the analysis by an experienced examiner (A.S.) who was blinded to the experimental conditions. All tissue specimens were analysed between 15 January and 15 December 2012.

### ***Histological analysis***

The tissue samples were fixed in 4% paraformaldehyde for 24 h, embedded in Tissue Tek (Sakura, Torrance, CA, USA) and sectioned in a plan parallel to the long axis of the tooth at a thickness of 4  $\mu\text{m}$  and then mounted on slides. The sections were stained with haematoxylin and eosin using standard procedure or prepared for molecular analysis. The images were captured by a digital camera (Nikon, Tokyo, Japan) interfaced with a light microscope (Olympus, Tokyo, Japan).

### ***Immunohistochemical analysis for IL-1 $\alpha$ , TNF- $\alpha$ , CXCL5 and IFN- $\gamma$***

The sections were stained using the kit for immunohistochemistry according to the manufacturer's instructions (Abcam, Cambridge, MA, USA). The primary antibodies rabbit polyclonal anti-human IL-1 $\alpha$  IgG (ab7632), mouse monoclonal anti-human TNF- $\alpha$  IgG1

[P/T2] (ab9579), mouse monoclonal anti-human CXCL5 IgG1 [MMO216-10L19] (ab89325) and rabbit polyclonal anti-human IFN- $\gamma$  IgG (ab7812) [all purchased from Abcam and all diluted 1:75 in phosphate-buffered saline (PBS)] were added and incubated overnight at +4°C. The antibodies were visualized with the peroxidase detection system using a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG Fc (ab97265; Abcam) and a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG H&L (ab6721; Abcam) and diaminobenzidine (DAB substrate kit ab94665; Abcam) as the contrasting chromogen. After staining, sections were counterstained with hematoxylin for 30 s, washed and mounted, and images were acquired using a microscope (Olympus) equipped with a digital camera (Nikon). For each antibody, cytoplasmic staining was recorded. The proportion of positive cells relative to the total number of cells (yielding the percentage of positive cells for each tissue sample) was calculated.

#### ***Immunofluorescence analysis for cluster of differentiation 14***

The sections were washed in PBS (pH 7.2), incubated with 5% bovine serum albumin for 60 min at room temperature and stained with mouse anti-cluster of differentiation 14 (anti-CD14)-fluorescein isothiocyanate conjugate (Clone M5E2; BD Pharmingen, San José, CA, USA) diluted 1:100 in PBS (Becton-Dickinson, San José, CA, USA) overnight at 4°C. After incubation, the sections were washed with PBS, incubated with Hoechst33342 for 7 min to stain the nucleus and the images were acquired with a fluorescence microscope (exposure time 18-20 ms) attached to a digital camera. Negative control were those with primary antibody omission.

#### ***Cytofluorimetric analysis for TRAP***

The unfixed specimens were digested in 4 mg/ml collagenase and 2 mg/ml dispase in a tube of 15 ml for 1h at 37°C with gently shaking. After digestion, the samples were filtered with 70  $\mu$ m stainer filters, and stained using the Fix & Perm kit for intracellular staining (Invitrogen, Monza, Italy). Briefly, cells were fixed with paraformaldehyde (Solution A) for

15 min at room temperature, washed in PBS and incubated with permeabilization solution (Solution B) and primary antibody (1 : 100 dilution ) for 30 min at room temperature. Then, the samples were washed and incubated with Solution B and secondary antibody at stock concentration for 20 min at room temperature. Primary antibody was mouse anti-human TRAP (Abcam) and secondary antibodies were anti-mouse-fluorescein isothiocyanate or anti-mouse-phycoerythrin conjugates (Abcam). The isotype control antibody was anti-mouse IgG2 (ab170192; Abcam). Samples were examined by FACS Aria II flow cytometry cell-sorting (Beckton-Dickinson). Data were analysed using FACS Diva Software 6.1.

### ***Clinical measurements***

The following clinical parameters were assessed by the same calibrated and masked investigator (G.M.M.) in the treated sextants on the day of surgery (i.e.  $T_0$ ): presence/absence of plaque at six sites/tooth; presence/absence of bleeding on probing at six sites/tooth; probing depth (measured as the distance in millimetres from the free gingival margin to the base of the pocket/sulcus) at six sites/tooth; gingival recession (measured as the distance in millimetres between the gingival margin and cemento-enamel junction) at six sites/tooth; and 5) clinical attachment level (measured in millimetres from the cemento-enamel junction to the apical point of the pocket/sulcus) at six sites/tooth. In addition, during the surgical session, the distance in millimetres between the cemento-enamel junction and the bone crest (BC) and the distance in millimetres between the BC and the bottom of the defects (BD) were recorded before ( $BC_0$ -BD) and after bone remodelling ( $BC_1$ -BD).

### ***Statistical analysis***

A statistical software program (SAS version 9.2; SAS Institute Inc, Cary, NC, USA) was used for data analysis. Only clinical measurements at the deepest site of the intrabony defects selected for tissue sampling (experimental sites) were included in the present calculations. Mean values  $\pm$  SD or median (interquartile range) were calculated for each variable using the subject as the experimental unit. The Shapiro–Wilk test and Q-Q normality plots were applied

to verify the normal distribution of the continuous variables. Differences between the two treatment procedures (FibReORS and ORS) at  $T_0$  and  $T_1$  (suture removal) were analyzed using the parametric Student's  $t$ -test for paired observations for data normally distributed (clinical data) or the Wilcoxon test for data not normally distributed (biomolecular data). The number of sites with bleeding on probing and the number of plaque-positive sites were compared between groups with the chi-square test. Values of  $p < 0.05$  were considered statistically significant.

### **Power Calculation**

A power calculation was done after the completion of the study, assuming the difference in TRAP expression between FibReORS and ORS-treated sites as a primary outcome variable. This analysis indicated that the study had a  $> 90\%$  power to detect a  $3\%$  difference between groups in TRAP expression, adopting an  $\alpha = 0.05$ .

## **RESULTS**

All the experimental sites were located at the interdental area between second premolar and first molar or between first and second molars. Periodontal parameters assessed at the sampling sites are shown in Table 1. No statistically significant difference was detected for any of the baseline characteristics between groups ( $p > 0.05$ ).

The amount of intrasurgical ostectomy was larger in the ORS sites sampled than in the FibReORS sites sampled ( $1.8 \pm 0.7$  mm vs.  $0.5 \pm 0.4$  mm,  $p < 0.0001$ ). Early wound healing was uneventful in both groups. At the time of suture removal, slight/moderate oedema in the surgical area was recorded in ten (76.92%) and in six (46.15%) of the ORS- and FibReORS-treated sextants, respectively. At the same time point, interproximal fibrin deposits were visible in eight (61.54%) of the ORS-treated sextants compared with two (15.39%) of the FibReORS-treated sextants.

Histologically, similar features were observed at  $T_0$  between ORS and FibReORS tissue samples, whereas a different healing pattern was detected at  $T_1$ . As expected, gingival tissues

from patients with chronic periodontitis exhibited an epithelium that varied in thickness and sometimes was hyperplastic and acanthotic. In the underlying connective tissue, a moderate-grade inflammatory cell infiltrate was detected (Fig. 1A,B). At  $T_1$  in the FibReORS biopsies the epithelium-connective tissue architecture was maintained, and the epithelium was layered and keratinized (Fig. 1C). The rete pegs had a normal shape, with the connective tissue containing a small mononuclear cells infiltrate. By contrast, ORS-treated sites exhibited a fully breakage of the epithelial tissue architecture. The epithelium was very thin (one or two cell layers) without any sign of ongoing keratinization. The reticular connective tissue did not display any evidence of fibres organization and lacked connective papillae projecting into the epithelium. A marked infiltration of mononuclear inflammatory cells was evident (Fig. 1D).

As shown in Table 2, expression of inflammatory mediators, particularly IL-1 $\alpha$  (Fig. 2) and TNF- $\alpha$  (Fig. S1), was statistically higher in the ORS biopsies than in the FibReORS biopsies at  $T_1$  ( $p < 0.001$ ). No significant differences were detected at  $T_0$ . At  $T_0$  faint immunostaining was observed in both groups for all mediators (Figs. S2 and S3).

Regarding the immunofluorescence analysis, a similar number of CD14-positive cells (Fig. 3), belonging to monocyte lineage, was found in both groups at baseline. By contrast, significantly greater CD14 positivity was observed at  $T_1$  in the ORS biopsies compared with FibReORS biopsies ( $p < 0.005$ ). In the ORS sites, such monocytes, as underlined by the significantly enhanced postsurgical cell expression of TRAP at  $T_1$  compared with  $T_0$  values ( $2.7 \pm 1.6\%$  vs.  $6.1 \pm 1.8\%$ ,  $p < 0.001$ ), were differentiating into osteoclasts (Fig. 4). Conversely, in the FibReORS biopsies (Fig. 4) no statistically significant changes in the mean TRAP expression levels were observed between  $T_0$  ( $2.9 \pm 1.5\%$ ) and  $T_1$  ( $2.6 \pm 1.6\%$ ). Differences between groups were statistically significant only at  $T_1$  ( $p < 0.001$ ).

## **DISCUSSION**

FibReORS has been proposed since 2007 as alternative to traditional ORS surgery in treating residual shallow/moderate intrabony defects at posterior teeth (5-7). The advantage of this

technique is reduction in the amount of resected bone in order to obtain defect elimination and positive bone architecture (5). This procedure is based on the histological observation that residual connective tissue attachment inserted in the root surface is able to prevent the apical migration of epithelial cells, thus limiting further bone loss during periodontal healing (28,29). Until now, no study has focused on the expression of inflammatory and osteoclastic activity markers in the early healing phase following FibReORS and ORS procedures.

In the present study, the consistency between histological and molecular data regarding the monocyte-osteoclast lineage differentiation process, along with the distinctive immunohistochemical inflammatory profile, suggest a more pronounced host response and delayed healing in the ORS-treated sites compared with the FibReORS-treated sites. In the FibReORS gingival biopsies collected at  $T_1$ , the epithelium displayed keratinization, the rete pegs had a normal shape and the reticular connective tissue appeared in active reorganization. These data are in line with previous findings on wound-healing mechanisms following flap surgery (30,31). By contrary, at the same time point, the tissue specimens from ORS-treated sites had not yet recovered the normal histological features of both epithelium and connective tissue. ORS sites heal by secondary intention in the interproximal area. This could partly account for the delayed wound-healing process (29). In addition, histologic and immunofluorescence studies in humans have demonstrated that, in presence of an inflammatory process, alterations of gingival epithelia may occur (32,33).

Whereas at  $T_0$ , faint immunostaining was detected in both groups, a higher postoperative increase in the expression level of all the investigated inflammatory mediators was observed in the ORS-treated sites compared with the FibReORS-treated sites. This was remarkably evident for TNF- $\alpha$  and IL-1 $\alpha$ . Although IL-1 $\alpha$  as an epidermal cytokine, a wide variety of other cells only upon stimulation transcribe the IL-1 $\alpha$  genes and produce the precursor form of IL-1 $\alpha$ , such as fibroblasts (12). Shortly after tissue injury, the secretion of IL-1 $\alpha$  stimulates the release of TNF- $\alpha$  in the skin and mucosae, in which mast cells appear to be the

predominant source of pre-formed TNF- $\alpha$  (34). Interestingly, surgical trauma and bone remodelling may stimulate prolonged production of IL-1 $\alpha$  and TNF- $\alpha$ . Reinhardt *et al.* (9) found a significant increase of IL-1 expression following surgical periodontal therapy. It has been demonstrated that a small amount of IL-1 is necessary for host defense and wound healing, particularly in a challenged environment (35), whereas overproduction of IL-1 may have a detrimental effect during the first phase of periodontal wound healing (36,37).

CXCL5 is another central mediator of inflammatory response, whose production is stimulated in keratinocytes and monocytes by the primary mediators TNF- $\alpha$  and IL-1 $\alpha$  and is inhibited by IFN- $\gamma$  (11). It is noteworthy that, whereas in FibReORS tissue specimens the postoperative levels of all the inflammatory mediators were equally low in all patients, in the ORS group CXCL5 and INF- $\gamma$ , although the levels of CXCL5 and INF- $\gamma$  were high, they were lower than those of IL-1 $\alpha$  and TNF- $\alpha$ . It is known that INF- $\gamma$  is an anti-inflammatory mediator and a molecular antagonist of CXCL5. Thus, the increased release of INF- $\gamma$  probably down-regulated the expression of CXCL5. Previous studies reported that the concentration of INF- $\gamma$  in gingival tissues is higher in active than in inactive sites (38,39).

The contribution of IFN- $\gamma$  in bone remodeling has not yet been fully clarified. Despite studies that have suggested inhibitory effect of IFN- $\gamma$  on RANKL-associated osteoclastogenesis and bone remodelling (40), it has also been shown that IFN- $\gamma$ -positive T-helper 1 cells are strongly associated with enhanced alveolar bone loss during periodontal infections (41,42) and that RANKL is often highly coexpressed in periodontal microorganism-reactive T-helper 1 cells (43). In addition, RANKL can indeed play an indirect role. The increased levels of IFN- $\gamma$  favour the phagocytic activity of PMNs and stimulate the monocyte response to lipopolysaccharide, enhancing production of IL-1 and TNF- $\alpha$  which promotes the breakdown of soft and hard tissue of the periodontium (44,45).

The differentiation of inflammatory mononuclear cells into osteoclasts is a key process in

bone loss associated with inflammatory conditions (23). In a previous histologic study Wilderman *et al.* reported that bone resorption after ORS procedures was delayed and occurred after 2 weeks of healing (46). In the present investigation an increased number of CD14-positive mononuclear cells was detected at  $T_1$  examination in all the ORS-treated sites. This finding had to be interpreted in the light of cytofluorimetric data showing a parallel increase in expression of TRAP. In contrast, FibReORS sites presented nearly unchanged numbers of CD14-positive cells along with slightly decreased levels of TRAP. Taken together, these findings might be interpreted as being an early phase of the osteoclast differentiation process. This phase involves the acquisition, by mononuclear osteoclast precursor cells, of specific phenotypic markers of mature osteoclast, such as calcitonin receptor, vitronectin receptor and TRAP (47,48). The role of osteoclastic TRAP is not fully understood, but studies on TRAP knock-out mice showed disturbed endochondral ossification with decreased resorptive activity of osteoclasts (49), whereas overexpression of TRAP was associated with increased bone turnover (24).

It is interesting to emphasize that CD14 is a co-receptor for bacterial lipopolysaccharide. Although lipopolysaccharide is considered its main ligand, CD14 also recognizes other pathogen-associated molecular patterns such as lipoteichoic acid (25). It is possible to hypothesize that differences in the amount of bacterial tissue penetration between the two treatment modalities could contribute to the higher expression of CD14 and IL-1 $\alpha$  in the traditional osseous resective surgery.

This study has some limitations. Tissue specimens were harvested just before ( $T_0$ ) and 10 days ( $T_1$ ) after the osseous resective surgical session. This was designed for ethical reasons. Another aspect to be addressed is the limited sample size. The present investigation was designed as a preliminary study. However, it is important to point out that, in spite of the small sample size, results are consistent. An advantage is the split-mouth design, which allowed for a comparison of the host inflammatory reaction between different tooth regions

within the same subjects.

In conclusion, the retention of the supracrestal connective fibres would seem to limit the inflammatory reaction, as well as the osteoclast-mediated bone resorption in the early wound healing phase following osseous resective surgery. Overall, all the mentioned findings appear to provide the biological rationale for the use of FibReORS, which has been clinically demonstrated to be a more conservative surgical approach than ORS, with the advantage of faster healing, minimal post-operative local side effects and patient morbidity. Further studies are needed to confirm the clinical relevance of these findings.

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## Figure Legends

**Fig. 1** Histological pictures representative of the tissue sections used in the analysis (hematoxylin and eosin staining, original magnification: x100). Gingival biopsies at baseline ( $T_0$ ) (**A, B**) show stratified squamous epithelium (arrow with solid line) with rete pegs and connective tissue papillae (arrow with dashed line). Gingival biopsies 10 d after surgery ( $T_1$ ) (**C, D**). In the osseous resective surgery with gingival fibre retention (FibReORS) biopsy (**C**) the tissue architecture is maintained, the epithelium is layered and keratinized (arrow with solid line), and the reticular component of the connective tissue is undergoing an active reorganization (arrow with dashed line). The osseous resective surgery (ORS) biopsy (**D**) exhibits a very thin epithelium without any sign of keratinization, a disorganized connective tissue with large amount of inflammatory cells and lack of connective papillae (arrow with solid line).

**Fig. 2** Immunohistochemical staining of tissue sections for interleukin-1 $\alpha$  (IL-1 $\alpha$ ) at baseline ( $T_0$ ) (**A, B**) and at the time of suture removal ( $T_1$ ) (**C, D**). Tissue treated with osseous resective surgery (ORS) exhibits a marked increase in the number of IL-1 $\alpha$ -positive cells (arrow with dashed line) (**D**) compared with tissue treated with osseous resective surgery with gingival fibre retention (FibReORS) (arrow with solid line) (**C**). All images original magnification: x100.

**Fig. 3** Detection of monocytes/macrophages in biopsies by measuring immunofluorescence following incubation with anti-CD14-fluorescein isothiocyanate conjugate (green) and counterstaining with Hoechst3334 (blue) (**A, B**) Biopsies at baseline ( $T_0$ ) (**C**) Biopsy at 10 days after surgery ( $T_1$ ) [osseous resective surgery with gingival fibre retention (FibReORS)]. No difference in signal intensity was found when compared with  $T_0$ . (**D**) Biopsy at  $T_1$  [after osseous resective surgery (ORS)]. Mapping of the fluorescence was highly positive, showing a large infiltrate of monocytes/macrophages. All images: original magnification: x200.

**Fig. 4** Cytofluorimetric analysis for tartrate-resistant acid phosphatase (TRAP) at baseline

( $T_0$ ) (**A, B**) and at 10 days after surgery ( $T_1$ ) (**C, D**). Increased levels of expression of TRAP were observed in the osseous resective surgery (ORS) biopsies, but not in osseous resective surgery with gingival fibre retention (FibReORS) biopsies at  $T_1$  compared with  $T_0$ .

### Supplemental Figure Legends

**Fig. S1** Immunohistochemical TNF- $\alpha$  staining (original magnification: x100) in gingival tissues from ORS and FibReORS. TNF- $\alpha$  was weakly positive at baseline in both treatment groups (**a-b**). ORS biopsies exhibit a marked increase in TNF- $\alpha$  positive cells at the time of suture removal (arrow with dashed line) (**d**) when compared to FibReORS sites (arrow with solid line) (**c**).

**Fig. S2** Immunohistochemical INF- $\gamma$  staining (original magnification: x200) in gingival tissues from ORS and FibReORS sites. INF- $\gamma$  was marginally positively expressed at baseline in both treatment groups (**a-b**), while a higher expression was observed at suture removal in the ORS biopsies (arrow with dashed line) (**d**) when compared to FibReORS sites (arrow with solid line) (**c**).

**Fig. S3** Immunohistochemical CXCL5 staining (original magnification: x200) of tissue sections at baseline (**a-b**) and at the time of suture removal (**c-d**). ORS biopsies exhibit a marked increase in the number of CXCL5-positive cells (arrow with dashed line) (**d**) when compared with tissues from FibReORS sites (arrow with solid line)(**c**).

**Table 1. Baseline ( $T_0$ ) clinical parameters at sampling sites of the study groups**

	<i>FibReORS</i> (n=26)	<i>ORS</i> (n=26)	<i>Differences between FibReORS and ORS</i> (p value)
<b>Bleeding on probing</b>	10/26	9/26	NS*
<b>Presence of plaque</b>	7/26	6/26	NS*
<b>Probing depth (mm)</b>	5.8 ± 1.2	5.4 ± 0.7	NS†
<b>Recession (mm)</b>	0.6 ± 0.9	0.4 ± 0.7	NS†
<b>Clinical attachment level (mm)</b>	6.4 ± 1.1	5.8 ± 0.8	NS†
<b>Ostectomy (mm)<sup>a</sup></b>	0.5 ± 0.4	1.8 ± 0.7	<0.0001†
<b>BC<sub>0</sub>-BD (mm)</b>	1.7 ± 0.7	2.1 ± 0.7	NS†
<b>BC<sub>1</sub>-BD (mm)</b>	1.1 ± 0.4	0.2 ± 0.3	<0.0001†

<sup>a</sup>Ostect

omy was the difference between the distance from alveolar bone crest to cemento-enamel junction calculated before and after bone remodelling; BC<sub>0</sub>-BD, distance between the alveolar bone crest and the bottom of the intrabony defect before remodelling; BC<sub>1</sub>-BD, distance between the alveolar bone crest and the bottom of the intrabony defect after remodelling; FibReORS, osseous resective surgery with gingival fibre retention; NS, difference between groups is not statistically significant ( $p > 0.05$ );

ORS, osseous resective surgery.

\*Chi-square test.

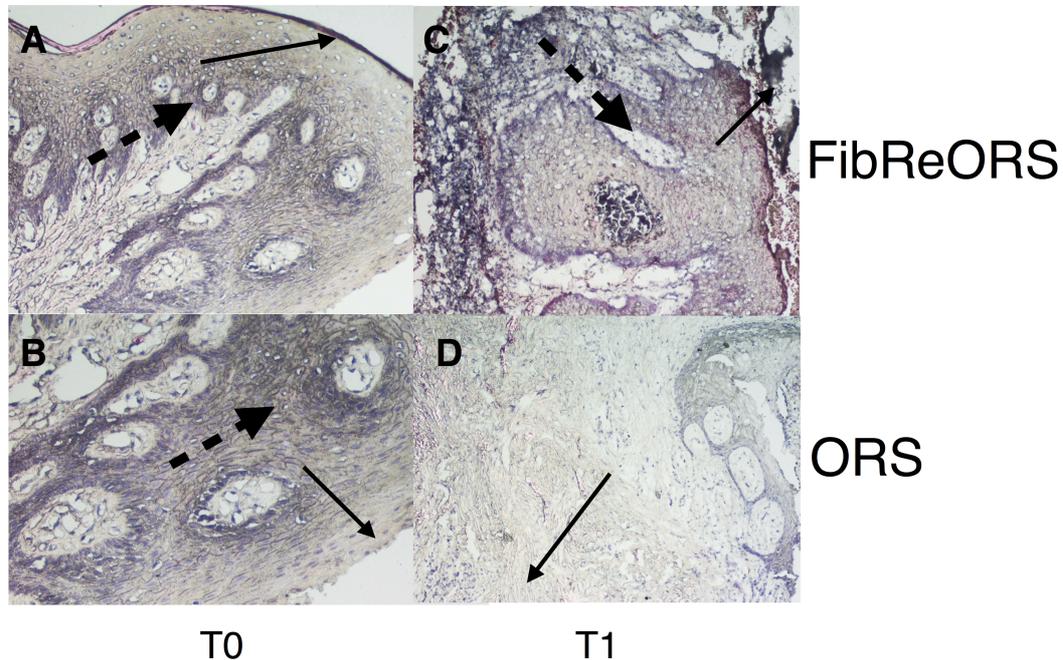
†Paired *t*-test; Data are reported as mean ± SD.

**Table 2. Postoperative ( $T_1$ ) percentage (%) of positive cells for inflammatory markers with respect to the total area occupied by the inflammatory infiltrate at sampling sites of the study groups**

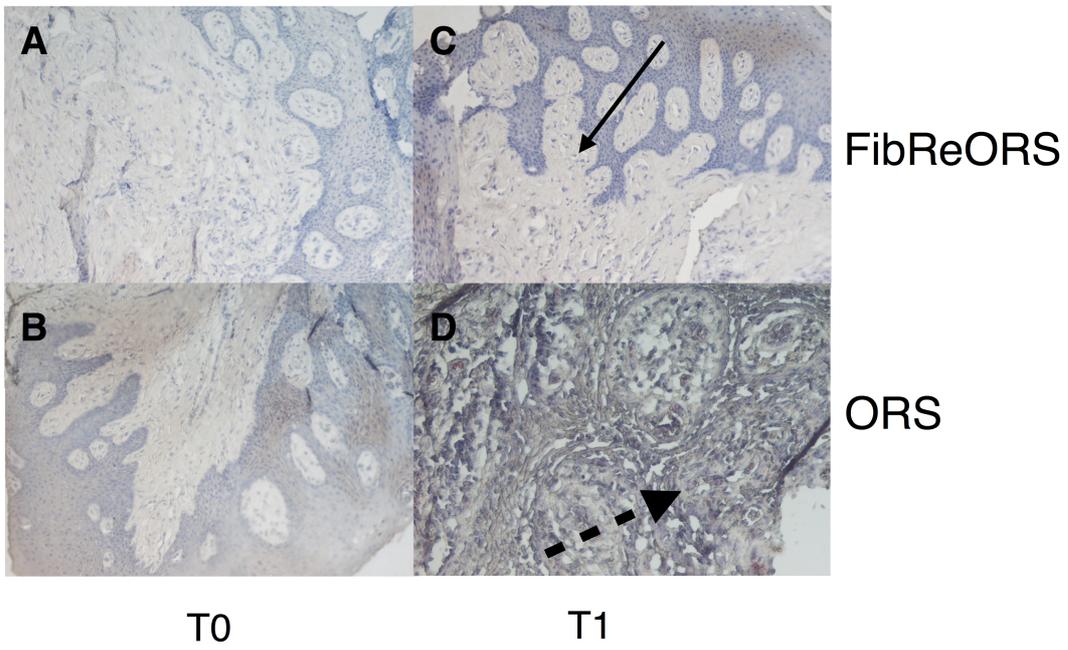
	<i>FibReORS (n=13)</i>		<i>ORS (n=13)</i>		<i>Differences between FibReORS and ORS</i>
	Mean $\pm$ DS	Median (IQ)	Mean $\pm$ DS	Median (IQ)	
<b>IL-1<math>\alpha</math></b>	6.14 $\pm$ 3.13	6 (3-10)	45.71 $\pm$ 7.80	43 (40-48)	<0.0001*
<b>TNF-<math>\alpha</math></b>	5.57 $\pm$ 2.37	5 (4-8)	48.29 $\pm$ 6.65	50 (42-55)	<0.0001*
<b>CXCL5</b>	6.86 $\pm$ 3.29	7 (3-10)	28.14 $\pm$ 9.53	31 (19-35)	<0.001*
<b>IFN-<math>\gamma</math></b>	7.00 $\pm$ 2.38	6.5 (5-10)	32.71 $\pm$ 8.75	33 (23-42)	<0.001*

IL-1 $\alpha$ , interleukin-1 $\alpha$ ; IQR, interquartile range; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; CXCL5, chemokine, C-X-C motif chemokine ligand 5; IFN- $\gamma$ , interferon- $\gamma$ .

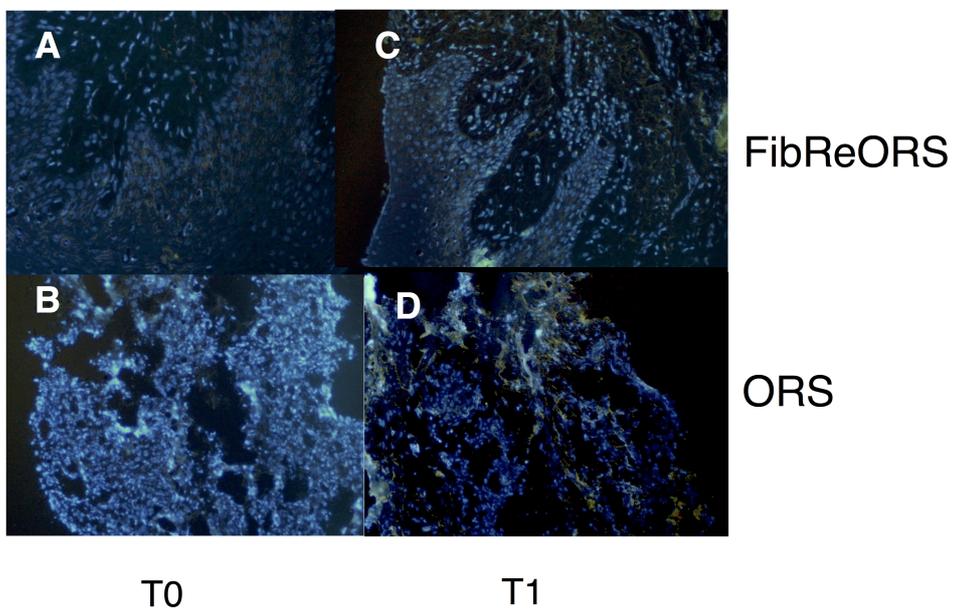
\* Wilcoxon-test.



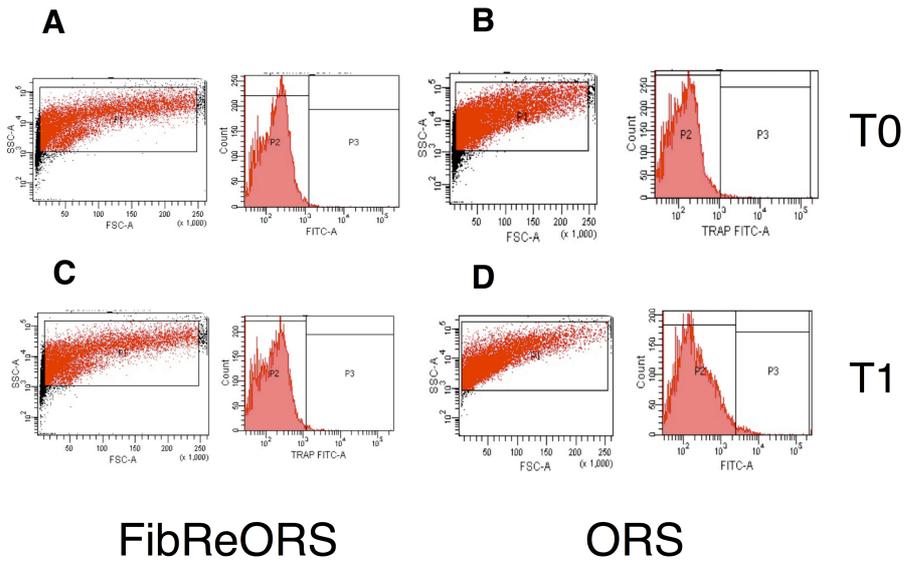
**Fig. 1**



**Fig. 2**



**Fig. 3**



**Fig. 4**