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**TESI DI DOTTORATO**

**Alveolar socket preservation technique: histological healing of hard  
and soft tissues.**

DOTTORANDO: Dott.ssa Gaia Pellegrini

MATRICOLA: R08442

TUTOR: Dott.ssa. Dellavia Claudia Paola Bruna

COORDINATORE DEL DOTTORATO: Prof. Roberto Weinstein

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*Questo percorso di crescita nasce in Michigan.*

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## **Post-extractive alveolar socket: macroscopic and volumetric changes**

### *Alveolar bone*

Alveolar bone is the process of the mandibular and maxillary bones that includes the dental alveolus, periodontal ligament and teeth. This anatomical structure is composed by the cortical lamina that covers the external side, the cortical lamina that line the alveolar socket named *lamina cribra* or bundle bone and the medullary space between the two laminas. The bundle bone is strictly related to the radicular surface of the tooth and gives attach to the periodontal ligament fibers that insert with a mineralized edge (Sharpey's fibers). At the coronal side of the alveolar ridge, this lamina continues with the external cortical bone and on the buccal side forms part of the alveolar crest.

Following tooth extraction and the interruption of the masticatory function and loading, the alveolar socket is filled by blood clot that starts the healing process, and the alveolar bone undergoes a remodelling phase resulting in a 3-dimensional alteration. As the alveolar bone development is related to the tooth formation and eruption, the loss of the natural dentition induces the alveolar ridge atrophy and volumetric reduction.

### *Healing of the alveolar socket*

The fate of buccal and lingual bony walls occurring after tooth extraction, and the phases of soft and mineralized tissue formation and maturation in the wound area were evaluated in several histological animal studies that performed bucco-lingual sections of healing sockets at different timepoints (Araujo & Lindhe 2005) (Cardaropoli et al. 2003). Within the first week after tooth extraction the bundle bone and the periodontal ligament are partially dissolved. The blood clot that immediately after extraction completely fills in the alveolus is progressively replaced by granulation tissue and provisional matrix composed of a newly formed highly vascularized, cellularized and not mineralized tissue. Several osteoclasts and multinucleated cells harbor in the residual tissue adjacent the healing socket indicating the coming remodelling process.

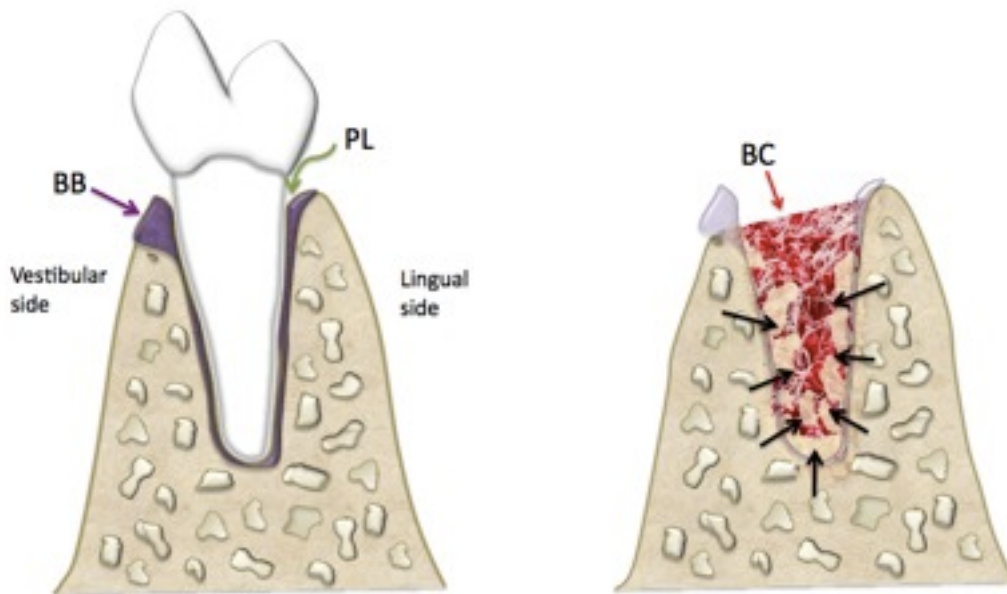


Figure 1: Schematic image of alveolar bone changes and socket healing immediately after tooth extraction. The bundle bone (BB) and periodontal ligament (PL) completely dissolve few days after extraction. The blood clot (BC) that fills the wound space, is colonized firstly by the inflammatory infiltrate and then by cells assigned to remodelling and maturation processes. The remaining bone tissue provides the cells and induce the regeneration process.

At two weeks this process was observed to proceed in the alveolus from the residual lateral and apical bone walls to the inner area (Figure 1). A thin layer of newly formed mineralized tissue with densely packed osteoblasts lined the residual bone enclosing the residual provisional matrix in the central compartment. Otherwise, the outer surface of the alveolar walls was not involved in the blood clot formation and stabilization, and appeared covered by a large number of osteoclasts. At a subsequent timepoint (4 weeks) the woven bone occupied most of the healing area and the provisional matrix was limited to a small central residue. The immature bone appeared in remodelling process and few marrow spaces were firstly observed at the periphery of the maturing tissue. The maturation of this newly formed tissue was observed also at 8 weeks after extraction. At this timepoint the alveolus was occupied by bone marrow and closed coronally by a bridge of lamellar and woven bone. A large number of osteoclasts was on the outer surface of the buccal bone, otherwise a limited number of cells was found lining the outer surface of lingual bone. At the conclusion of the remodelling and maturation process (120-180 days in dog) the experimental site appeared covered by a “new cortical lamellar bone”

that lined a layer of woven bone. The inner area was filled by thin and few trabeculae of lamellar bone, and bone marrow contained a large number of adipocytes and few inflammatory cells (Araujo & Lindhe 2005) (Cardaropoli et al. 2003).

The observation reported above derive from pre-clinical studies conducted on dog model and should be adjusted on the human healing pattern. The healing pattern in dogs is about 1.5 times faster than in human and these two species present some differences related to the biological potential, however the consistency between these two models may justify the use of this pre-clinical model to explore biological aspect related to the wound (Pellegrini et al. 2011, Giannobile et al. 1994). Histomorphometrical analyses on alveolar socket healing in human were performed on cadavers (Boyne 1966, Amler 1969) or on tissue biopsies harvested using a trephine bur at different times after tooth extraction and during the implant bed preparation (Carmagnola et al. 2003, Trombelli et al. 2008, Rasperini et al. 2010).

Trombelli et al. (2008) described the healing of the human extraction socket at early (2-4 weeks), intermediate (6-8 weeks) and late (12-24 weeks) timepoints. The authors observed a slow reparative and regenerative process with high interindividual differences. During the first weeks the granulation tissue replaced the blood clot. The vascular density and macrophages number picked and tended to decrease in the following weeks when the granulation tissue was replaced by provisional matrix and woven bone. The deposition of this first immature bone corresponded to the increased number of osteoblasts observed within the samples. At the late timepoint the immature mineralized tissue was still in modelling and remodelling phase, however only in 1 of the 11 patients the mature lamellar bone and bone marrow were detected. A complete maturation of the tissue with lamellar bone and large bone marrow spaces filling the extractive site was found 1 years after tooth extraction (Carmagnola et al. 2003).

#### *Radiographic findings and dimensional changes*

This tissue remodelling process that has been analyzed histologically corresponds to dimensional changes of the alveolar crest that play a key role in the clinical

practice. The quality and quantity of remaining bone tissue after tooth extraction has strong implications for the maintenance of aesthetics in anterior area and for the feasibility of implant placement. Since this clinical importance, several studies evaluated radiographically and clinically the dimensional changes of the alveolar crest. Nevins et al. (2006) investigated 3-dimensionally (by means of CT-scan) the fate of the buccal bone 3 months after extraction of anterior maxillary teeth and observed that only 29% of the investigated sites remained stable, and 71% showed a loss of more than 20% in height. Furthermore they observed a correlation between the height at crest width of 6 mm between the baseline and the endpoint (3 months post-operative). A further clinical and radiographic study performed on posterior maxillary sextants, confirmed the data of Nevins et al. and estimated a mean vertical bone resorption of 3.75mm at 3 months after tooth extraction (Crespi et al. 2009).

A recently published clinical trial (Farina et al. 2011) on dimensional changes in posterior maxillary areas reported that following tooth extraction the observed reduction of the bone height results both from the apical displacement of the alveolar crest as well as the coronal expansion of the sinus cavity. These morphological changes were mostly evident in correspondence to the molar region and less marked in the premolar sites.

Van der Weijden et al. (2009) reviewed the clinical phases of dimensional alveolar crest alterations and the factors affecting this process. From radiographic assessments, following tooth extraction the alveolus is filled with mineralized tissue for about 2.57 mm on average and the crestal height reduces vertically for about 1.59 mm. The vertical and horizontal ridge reduction mostly occur within the first 3 months and continues for almost 1 year. The smoking habit influences negatively the wound healing since it has cytotoxic and vasoactive effects, while the administration of chlorhexidine rinses demonstrated an increase in bone density. The surgical technique also seems to influence the clinical result: a mini-invasive procedure that limits the flap elevation and does not compromise the vascular supply may reduce the soft tissue recession and favor the bone maintenance (Van der Weijden et al. 2009).



### **Clinical implications of volumetric alveolar ridge reduction**

In the modern concept of implant rehabilitation the fixture should not be placed in the site with the best anatomical conditions (i.e. bone quality and quantity), but according to the needs of the prosthetic rehabilitation (i.e. emergence of the implant aligned to the crown). Unless the clinician performs a bone regenerative procedure (i.e sinus augmentation, alveolar crest augmentation...), the bone loss occurring following tooth extraction may compromise the correct implant placement or may cause the rehabilitation with prosthesis aesthetically unacceptable (Figure 2).

Moreover the loss of hard tissue is associated to reduction of keratinized tissue on lingual and buccal aspects of the edentulous area. Surgical procedures were proposed for peri-implant keratinized soft tissue augmentation, including: rotated palatal flap, graft of resorbable collagen membrane or connective tissue (Nemcovsky & Moses 2002) (Simion et al. 2012, Wiesner et al. 2010).

Based on guided bone regenerative approach, several biomaterials and procedures were introduced to prevent/limit the alveolar socket collapse (Figure 3) and preserve the patient from further and invasive regenerative surgeries including: I) the use of membrane alone or associated to filling materials as barrier against epithelial growth, II) the use of graft material as osteoconductive scaffold and space maintenance device, III) the use of biologics (growth factors and cell-based products) to induce the bone formation. Growth factors are molecules normally expressed during the wound healing and regulate fundamental cellular activities: the migration, proliferation and differentiation (Barrientos et al. 2008). Bone morphogenetic proteins (BMP) are a group of growth factors with a multitude of functions orchestrating the tissue architecture. In regenerative medicine these molecules were introduced to accelerate or induce bone formation.

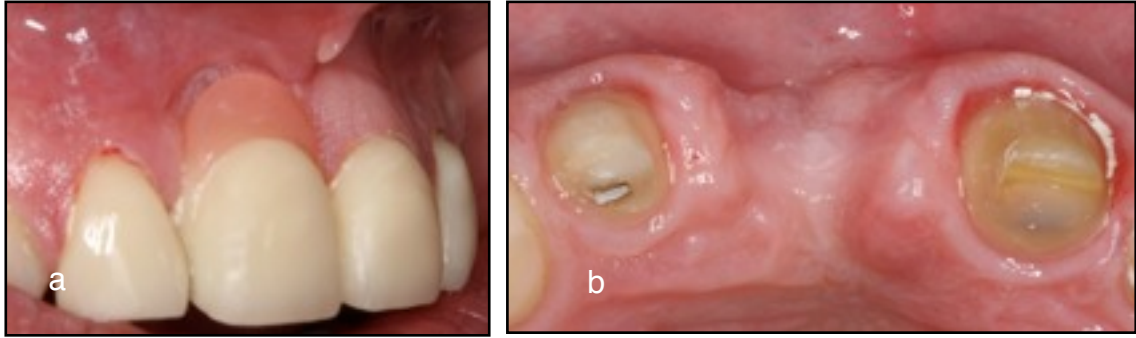


Figure 2: Following tooth extraction the hard and soft tissue underwent to a strong contraction that was initially masked with a resinous flange (a-c). A further reconstructive surgery was performed to increase the soft tissue volume (d, e).

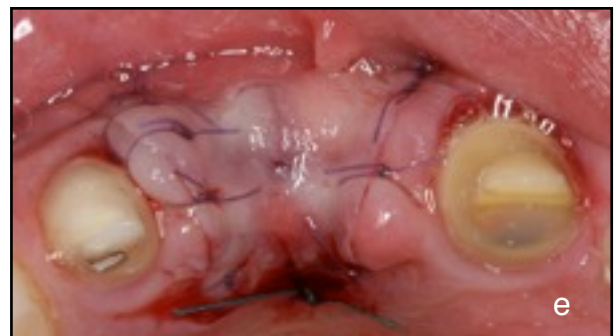




Figure 3: Following tooth extraction, a 3-dimensional contraction of the alveolar crest normally occurs (a). Augmentation procedure of post extractive alveolus (b) reduces the crestal bone loss.

#### *Filling materials for bone tissue augmentation*

Based on the biological properties, filling materials were classified as: I) osteogenic materials that enclose vital osteogenic cells, II) osteoinductive materials that maintain biomolecules able to attract the host osteogenic cells, III) osteoconductive materials that act only as scaffold for host cell colonization and grow. To date, autologous bone is the only osteogenic material and is considered the gold standard for regenerative procedures. However the need of a second surgical site to be harvested limit its use in daily practice and for this reason most of the clinical trials were designed with xenogenic or synthetic materials as graft.

Between osteoconductive materials, the hydroxyapatite is one of the most largely proposed in the literature since it forms up to 50% of human mineralized tissues. This mineral exists in many phases, with similar or identical chemical structures which may be arise different body response when grafted.

Non-resorbable hydroxyapatite (NHA) crystals was introduced for ridge preservation procedure in 1996 (Nemcovsky & Serfaty 1996). The Authors proposed this material after extracting maxillary anterior teeth associated to rotated pediculated split thickness palatal flap and at 12 to 24 months of follow up. They

observed a predictable reduction of the post-operative ridge deformation. Luczyszyn et al. (2005) evaluated histologically and clinically the effects of acellular dermal matrix graft (ADMG) with or without the resorbable hydroxyapatite (RHA, Aligpore) 6 months after tooth extraction. At re-entry surgery, the group grafted with ADMG+RHA resulted in a significantly greater ridge thickness preservation. The histological analysis revealed remnants of RHA mostly surrounded by a highly vascularized fibrous connective tissue (Luczyszyn et al. 2005). In a histomorphometric study, a nanocrystalline hydroxyapatite paste was used in extraction socket in dogs. The filling material was unable to prevent the dimensional ridge alteration and the alveolar wall resorption on the buccal and lingual aspects. From histological analyses, a high variability of NHA resorption and osteoconductive properties were observed both at 3 and 6 months after augmentation. At 3 months both osseointegrated and non-osseointegrated particles were enclosed by soft tissue and surrounded by macrophages and multinucleated giant cells, while at 6 months small non-resorbed particles were mostly covered with newly formed bone and only few single NHA particles were still encased by soft tissue (Rothamel et al. 2008).

Data from these studies seem to indicate the hydroxyapatite as useful material for graft procedure as result of its osteoconductive and biocompatible properties. However despite this mineral constitutes most of the mineralized body tissues, it was not completely remodelled and replaced by newly formed bone but small particles still remained at least 6 months after grafting.

The osteoconductive properties of xenograft materials and their advantages in alveolar socket preservation procedure were analyzed in several clinical and pre-clinical studies.

Artzi et al. (2000) designed a clinical and histomorphometrical study to evaluate the influence of cancellous porous bovine bone mineral (PBBM) on healing of human fresh extraction sockets. Nine months after healing the 82.3% of the extraction socket was mostly filled with woven bone and only apically with lamellar bone. PBBM remaining particles constituted about 30.8% of the newly formed tissue and decreased from the coronal to the apical side of the samples. The filling material appeared well incorporated in the generated socket osseous tissue that

represented about the 46.3% of the overall tissue and was more dense in the deeper (63.9%) than in the crestal region (15.9%). Data from this study confirmed the pattern of bone formation (from the apical to the coronal side) observed in studies on alveolar sockets that were let to heal spontaneously (Trombelli et al. 2008). Furthermore even at 9 months of healing the PBBM resulted not completely resorbed, but was osseointegrated in the newly formed bone. In a further histochemical study on the same samples the authors (Artzi et al. 2001) demonstrated the presence of osteoblasts within an osteoid layer and lining the interface zone of PBBM particles and the new osseous tissue. This study confirmed the osteoconductivity and promoting osseous ingrowth activity of the xenograft material. In a histological human study Carmagnola et al. (2003) divided 31 extraction sockets in three groups: group A, the extraction sockets were covered with a resorbable membrane and group B, the extraction sockets were filled with PBBM, group C sockets were left to heal spontaneously. Samples from group A showed large amounts of lamellar bone and bone marrow and small proportions of woven bone while sites grafted with PBBM were comprised of connective tissue and small amounts of newly formed bone surrounding the graft particles. Only 40% of the circumference of the PBBM particles was in contact with woven bone. Sites from group C were characterized by the presence of mineralized bone and bone marrow (Carmagnola et al. 2003). Animal (dog) studies described the early response of fresh post-extractive sites treated with xenograft filling material and compared the augmented sites with not treated sites. At earlier healing stages (2 weeks) grafted sockets demonstrated xenograft particles enclosed in connective tissue and coated by multinucleated cells while non-grafted sites already showed newly formed woven bone occupying most of the socket (Araujo et al. 2009). This response is typical of a foreign body reaction which can be elicited by the xenograft and though it is clinically non-immunogenic, non-toxic and chemically inert (Luttikhuisen et al. 2006), it results in a delayed healing response during the earliest stages of socket healing.

In the last decades the allograft material was introduced in regenerative dental practice.

It is generally used in one of two forms: freeze-dried bone allograft (FDBA) and demineralized freeze-dried bone allograft (DFDBA). The two types of graft materials work by different mechanisms. FDBA provides an osteoconductive scaffold and elicits resorption when implanted in mesenchymal tissues (Goldring et al. 1988). DFDBA provides an osteoconductive surface and a source of osteoinductive factors (Urist 1965). Therefore, it elicits mesenchymal cell migration, attachment, and osteogenesis when implanted in well-vascularized bone, and it induces endochondral bone formation when implanted in tissues that would otherwise not form bone.

For these positive features, DFDBA has been used for alveolar socket augmentation.

In a human histologic study, Becker compared the quality of the tissues within extraction sockets generated using different grafting materials or a mixture of grafting material and bone morphogenic proteins (hBMP) (Becker et al. 1998). Eight patients were treated with xenogenic bovine bone (n=5 sockets), DFDBA (n=3 sockets), autologous bone (n=3 sockets) harvested in the oral cavity, or a mixture of BMP in an osteocalcin/osteonectin carrier (hBMP/NCP) (n=2 sites). Three patients also received titanium screws fixed in extraction sockets treated with bovine bone (n=3 sites), DFDBA (n=2 sites) or intraoral autologous bone (n=1 sites). Biopsies were harvested between 3 and 6 months. Biopsies from bovine bone sockets revealed dead implanted particles surrounded by connective tissue. Isolated sections showed host bone in contact with the bovine bone particles. Screws placed in bovine bone were surrounded by connective tissue. DFDBA also showed dead particles encapsulated in dense connective tissue and screws placed in this material were surrounded by connective tissue. Autogenous bone resulted in a combination of new and non-vital bone with graft particles encapsulated in connective tissue. Autologous bone produced some contact of vital bone with the screw, although most of the screw surface was still encapsulated by connective tissue. hBMP/NCP treated sockets showed generation of woven and lamellar bone. According to the study's conclusions bovine bone, DFDBA, and intraoral autologous bone did not promote extraction socket healing, while sockets implanted with hBMP/NCP contained vital woven and lamellar bone. Xenogenic bovine bone and DFDBA did not contribute to bone to micro screw contacts and seem to be not recommended

for enhancement of vital bone to implant contacts. Intraoral autogenous bone also did not appear to significantly contribute to bone to implant contacts. Intraoral autologous bone, xenogenic bone, and DFDBA appeared to interfere with normal extraction socket healing (Artzi et al. 2001, Becker et al. 1998).

The efficacy and clinical benefits of augmentation procedures were assessed in several clinical and radiographic trials.

Nevins et al. (2006) observed the alveolar bone resorption of maxillary anterior post-extractive defects augmented with PBBM or not-augmented. Computed tomography evaluation was taken immediately after extraction and then 30-90 days after healing. The Authors demonstrated that more than 20% of the crestal height at 6 mm width occurred in 71% of the untreated control sites. In contrast, this percentage was only 16% in the PBBM test sites (Nevins et al. 2006). In a clinical study Rasperini found that the PBBM grafted in maxillary posterior post-extractive sites increase the probability of maintaining the alveolar ridge volume. The maintenance of the vertical dimension in this specific area also decreased the demand for a sinus augmentation procedure (Rasperini et al. 2010).

#### *Membranes in alveolar socket preservation techniques*

Guided bone regeneration (GBR) techniques utilize barrier membranes to refrain gingival cells from penetrating into the defect to be regenerated. The concept of compartmentalization was introduced by Melcher (Melcher 1976) to explain periodontal wound healing but it may not be applicable to socket healing. If it were, one would expect the socket to be filled with soft tissue in all instances. On the other side, even early observations in humans and animals demonstrated that the alveolar socket tends to heal by regeneration of bone up to the alveolar crest. As in periodontal wound healing (Wikesjo et al. 1992), the stability of the blood clot previously described explains why the compartmentalization concept does not result in a socket filled by epithelium and how epithelial cells migrate over the granulation tissue to close the healing socket. Questions remain as to whether barrier membranes have an effect in maintaining alveolar ridge morphology.

In 1997 Lekovic and coworkers adopted non-absorbable ePTFE membranes for the preservation of the alveolar ridge following tooth extraction. No changes in clinical

measures were noted in the test sites that remained protected for 6 months while significant volumetric changes were observed in control sites and in test sites experiencing membrane exposure (Lekovic et al. 1997). Pinho and co-workers evaluated the use of a titanium membrane with or without autologous bone graft. They found no significant differences between groups and therefore concluded that space maintenance is more important than the use of grafting materials in the treatment of extraction sockets (Pinho et al. 2006).

Barrier membranes seem to minimize alveolar bone resorption when compared to non-intact (released) periosteum regardless of the use of additional grafting material. Titanium membranes certainly would have a distinctly different mechanism of action when compared to resorbable membranes that on the other side reduce the potential of exposure and do not require a second surgical intervention for their removal. In 1998 Lekovic et al. examined the effect of glycolide and lactide polymers membranes demonstrating reduced loss of alveolar height, more internal bone socket bone fill and less horizontal resorption than controls (Lekovic et al. 1998). Luczyszyn evaluated the effect of acellular dermal matrix with or without a resorbable hydroxylapatite graft. Both groups preserved ridge thickness, although, better results were achieved in the combined treatment group suggesting that bone grafts might benefit bone regeneration when using a resorbable membrane (Luczyszyn et al. 2005).

A recent study performed a detailed evaluation of the healing of extraction sockets covered with a resorbable collagen membrane. Through the use of histological evaluation, subtraction radiography and of  $\mu$ -CT analysis, this study demonstrated that adequate bone formation for implant placement occurs as early as 12 weeks following tooth extraction, with insignificant changes in alveolar ridge dimensions (Neiva et al. 2011).

#### *Socket grafting and osteointegration*

As reported above, several human and animal studies observed only a partial resorption of the grafted particles at short and long timepoints (Artzi et al. 2001, Becker et al. 1998, Carmagnola et al. 2003). Doubts may be arisen on the achievement of the osteointegration of implants inserted in augmented sites and on the success of the restorative therapy. Histological animal studies (De Santis et al.



2011, Fiorellini et al. 2003) evaluated the osteointegration of dental implants following bone regeneration performed with different bone fillers and observed a bone-to-implant contact similar to that of implants placed in pristine bone (40% to 65%). Furthermore clinical studies observed that good primary stability can be reached at implant insertion, that the grafting procedure does not impair early osteointegration (Carmagnola et al. 2003) and that implants placed in bone regenerated using mineralized grafts are able to sustain loading and provide similar long-term results as those placed in pristine bone (Fiorellini et al. 2007).

#### *The importance of peri-implant soft tissue*

The post-extractive alveolar bone loss drives the alteration of the overlying soft tissue and leads to the reduction of the vestibular width of keratinized mucosa.

In this anatomical condition, following implant restoration the implant neck may be surrounded by thin non-keratinized alveolar mucosa. Questions were arisen on the association between the width of the keratinized tissue (KT) and the aesthetic outcomes or the long-term peri-implant health. In a clinical long term study, Wennstrom et al. (1994) examined 39 patients who had received a full-arch fixed bridge reconstruction > or = 10 years ago or a partial reconstruction > or = 5 years ago on a total of 171 implants. The authors observed that both the standard of plaque control and the health condition of the peri-implant mucosa were not influenced by the width of the keratinized mucosa and the mobility of the border tissue. In a further study the authors also observed that these conditions (lack of KT or mobility of the mucosa) were poor predictors of soft tissue recession occurring during the 2 years of follow-up, but indicated a greater amount of recession in women than in men, in the mandible than in the maxilla, at lingual than at facial sites and with increased initial probing depth (Bengazzi et al. 1996).

Kim et al. (2009) observed the peri-implant mucosal stability and the inflammatory parameters on a total of 276 implants for a follow-up of 13 months on average. The authors found that in cases with insufficient mucosa in the vicinity of implants, oral hygiene management, as well as the health condition of soft tissues, does not necessarily mediate adverse effects. However, the deficient keratinized mucosa has the risk of increasing gingival recession and marginal bone resorption. The authors

suggest that in cases requiring long-term maintenance management and esthetics, the presence of an appropriate amount of KT is required (Kim et al. 2009).

Otherwise Roos-Jansaker followed 218 patients treated with titanium implants for 9 to 14 years and reported the associated factors related to peri-implant lesions. It was observed that the presence of keratinized mucosa was associated to mucositis and bone loss. The authors hypothesized that sites without keratinized mucosa are more susceptible to soft tissue recession and are less prone to pocket formation (Roos-Jansaker et al. 2006).

As reported by the literature, the presence and amount of keratinized mucosa seems not influence the long-term implant survival. However the management of the peri-implant soft tissue is considered critical for the esthetic outcome. Studies on mucosal stability around dental implants reported a mucosa rearrangement following few months after restoration (Grunder 2000). Grunder et al. (2000) evaluated soft tissue stability in 10 single-tooth implants in the maxillary incisor area. All cases were treated following the same protocol which included: the guided bone regeneration performed with a non-resorbable membrane placed buccally at the implant placement time, and the KT augmentation by connective tissue grafting inserted buccally. One year after prosthesis insertion this author observed the soft tissue shrinkage on the buccal side of the implant crown of 0.6 mm on average. In addition the soft tissue volume in the papilla area increased on average by 0.375 mm.

In conclusion, as reported by an interesting review (Cairo et al. 2008), despite the lacking of scientific evidence on key role of KT for implant long term stability, the soft tissue management may be indicated in clinical situation with a challenge aesthetic outcome.

### **Rationale of the research**

As reported by literature that was reviewed above, both hard and soft tissues are determinant for the success of the implant therapy.

The volume and quality of mineralized tissue guide the treatment plan of the clinician that may propose a fixed implant supported restorative therapy as more comfortable alternative to a partial or total removable prosthesis. On the other hand the maintenance of the alveolar bone volume adopting easy and economic

augmentation procedures immediately after dental extraction may preserve the patient by further reconstructive therapies that result more invasive, expensive and with a less certain prognosis.

Furthermore the long-term success of the implant therapy depends by several factors: the prosthetic rehabilitation, a correct hygiene maintenance and the bone stability and aesthetic. Soft tissue is directly/indirectly related to each of these aspects and is taking even more importance in this kind of restoration.

From these considerations, aim of this human histological-clinical research was to describe histologically the morphological features of hard and soft tissues healed at different endpoints following post-extractive augmentation procedure and to observe the behavior of cellular population and molecular markers indicating the activity and quality of the bone and mucosa that will received an implant. These descriptive data were also integrated with clinical measurements on tissue volume and the need to perform further reconstructive therapies.

The research was organized as follow: i) firstly was analyzed the hard tissue formed after a regenerative procedure with bone substitute and bioabsorbable membrane was analyzed and clinical measures on volume maintenance were taken (Study A), ii) subsequently the soft tissue healing was analyzed following the same regenerative procedures or excluding the placement of the membrane, factor that may differently influence the tissue formation and maturation (Study B).

### **Study A: materials and methods**

Two clinical centres were selected to participate to this study. A total of 16 not-smoking patients (8 for each centre) who need the extraction of one periodontally compromised, maxillary molar tooth were included. All subjects satisfied the following entry criteria:

- Age more than 18 years
- Non existence of systemic disease, and metabolic bone disorders
- No current pregnancy

- No history of malignancy, radiotherapy, or chemotherapy for malignancy in the past 5 years
- Absence of periapical pathology or suppuration
- Full Mouth Plaque Score and Full Mouth Bleeding Score less than 25%
- No history of periodontal surgery in the experimental site

Informed consent was obtained from all subjects to be entered in the study.

#### *Randomization*

Each patient was the experimental unit and contributed with one extraction socket. All subjects were randomly assigned to one of the two treatment regimens. To assign the treatment, Central Registrar designed tables with a balanced random permuted block approach. The treatment for each patient was communicated to the operator immediately after tooth extraction. In order to reduce the chance of unfavourable splits between test and control groups in terms of key prognostic factors, the randomisation process will take into account the following variables: first or second molar site, presence of adjacent teeth.

#### *Clinical measurements*

Before tooth extraction an impression was taken with irreversible hydrocolloid material. Lab-Technician removed the extracted tooth from the cast and fabricated a stent as showed in figure 4, 5.

Measurements were harvested immediately after extraction and at 3 and 6 months after surgery. After positioning of the stent blind examiner recorded the following clinical parameters by means of periodontal probe PCP UNC 15 (Hu-Friedy): B/C (Buccal-Coronal), B/A (Buccal-Apical), B/O (Buccal-Occlusal), Center, P/O (Palatal-Occlusal), P/A (Palatal-Apical) and P/C (Palatal-Coronal).

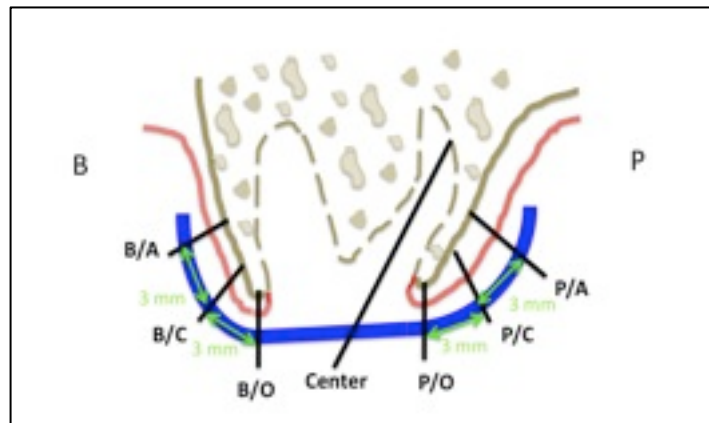


Figure 4: Scheme of alveolar socket after tooth extraction. A stent (blue line) was prepared with acrylic resin to enable the standardized introduction of the probe. Mini-tubes (from orthodontic use) (black lines) were included in B/A, B/C, P/A and P/C position allowing the passage of a periodontal probe. After tooth extraction, metal tubes (black lines) were slotted in the stent at B/O, Center and P/O position and fixed with acrylic resin. At baseline, 3 and 6 months after surgery the blinder examiner harvested the measures from the bone to the external surface of the stent.



Figure 5: Picture of experimental site, 6 month after extraction, with the stent in place.

### *Surgical procedure*

At the appointment before surgery, all patients received professional oral hygiene procedures and instructions to eliminate any infective complication. A micro-invasive procedure was used for tooth extraction and if necessary roots were separated with a bur and removed separately. A careful curettage of the socket was performed from the bottom up to the gingival margin with an accurate clean up of the sulcular epithelium. In test sites the alveolar socket was filled with a graft of Bio-

Oss Collagen (Geistlich Pharma, Switzerland) and in the control sites the alveolus was left untreated. Both test and control group sites were closed with a Gore-Tex non-resorbable 5-zero suture. At 6 month after surgery X-Rays were taken and implants were placed. According to Geurs (Geurs et al. 2001), the authors decided for implant placement with or without previous sinus floor augmentation. Basing on radiographic observation of the residual bone, sinus floor augmentation procedure was performed in sites where vertical residual bone was less than 8 mm.

#### *Statistical analysis*

Calibration of blind examiners was performed on triplicate measurements before the beginning of the study. The variability among the centres was evaluated with the intra-class coefficient of correlation. Results of test and control group and of sites missing and maintaining buccal plate were analyzed. The efficacy parameters were the decision for sinus augmentation before implant placement as determined by bone height evaluated at 6 months with X-Rays, and the changes in bone volume as determined by comparing B/C, B/A, B/O, Center, P/O, P/A and P/C measurements between baseline, 3 and 6 months. The end point differences between groups were analyzed by Wilcoxon Mann Whitney test. For the data showing the implant placement with or without previous sinus augmentation procedure the Odd Ratio has been calculated.

#### *Histologic and histometric analyses*

At 3, 6 and 9 months, immediately before implant placement, one bone histologic sample (2 mm long x 1 mm diameter) was harvested from 3 selected patients by means of a trephine bur.

The biopsied specimens were immersion fixed in 10% formalin/0.1M phosphate buffer saline (PBS) (pH 7.4) 24 hours at room temperature, then routinely dehydrated in increasing concentrations of ethanol (from 70 to 100%), infiltrated with agitation and vacuum and embedded in Kulzer Technovit 7200 VLC (Bio-Optica, Milano, Italy). Each block was then longitudinally cut in a buccal-lingual plane using a diamond saw (Micromet Remet, Bologna, Italy). The two central sections were ground and polished (LS2, Remet, Bologna, Italy) to a final thickness of 40 μm according to the technique described by Donath & Breuner (Donath &

Breuner 1982). Staining was performed with toluidine blue/ pyronine G (Sigma-Aldrich, St Louis, MO). The sections were view, photographed in a Nikon light microscope (Eclipse E600) equipped with a calibrated digital camera (DXM1200, Nikon, Tokyo, Japan) and analysed histomorphometrically using a point-counting technique procedure. A lattice comprising 100 test points was superimposed over each histologic section photographed at a total microscopic magnification of 40X. The tissue underlying each grid intersection was recorded as either new bone, residual Bio-Oss collagen or connective tissue/marrow spaces. The number of hits containing new bone, graft material and connective tissue/marrow were separately divided by the total number of possible intersections and thus expressed in percentage values representing the fraction area of these 3 histologic components.

### Study A: Results

#### *Study population*

Sixteen patients with average age of 54 years were included in the study. The distribution of teeth and defects at baseline is showed in table 1. In both test and control group 1 patient did not complete the 3 months evaluation and the terminal evaluation. The analyses at each endpoint were conducted on 6 subjects in the test group and 8 subjects in the control group. After tooth extraction, in 5 patients of the control group buccal wall was missing (BWM) and only 4 patients of the same group preserved the entire alveolar bone (four-wall defect). In the test group all patients maintained the entire alveolar bone after tooth extraction.

	Control Group (n=9)	Test Group (n=7)
Tooth:		
I Molar	5	5
II Molar	4	2
Buccal wall:		
Maintained	4	7
Not-Maintained	5	0

Table 1: Distribution and characteristics of defects at baseline.

### *Histological evaluations*

Histological evaluations were performed on samples harvested at 3, 6 and 9 months. By 3 months (Figure 6) a great amount of PBBM particles are surrounded by provisional matrix through the entire tissue sample. Provisional matrix is composed by highly cellular, fibrous connective tissue ongoing in mineralization process. No inflammatory infiltrated is detectable in connective tissue and surrounding the bone particles. Implanted material displayed resorption areas with lacunae and osteoclast-like cells, reflecting osteoclast activity (Figure 7). Some Bio-Oss Collagen particles appear colonized by cells. At the observation with polarized light, the provisional matrix shows area with a more advanced degree of mineralization process. No newly formed bone is detectable, however residual bone appear on apical portion of the sample.

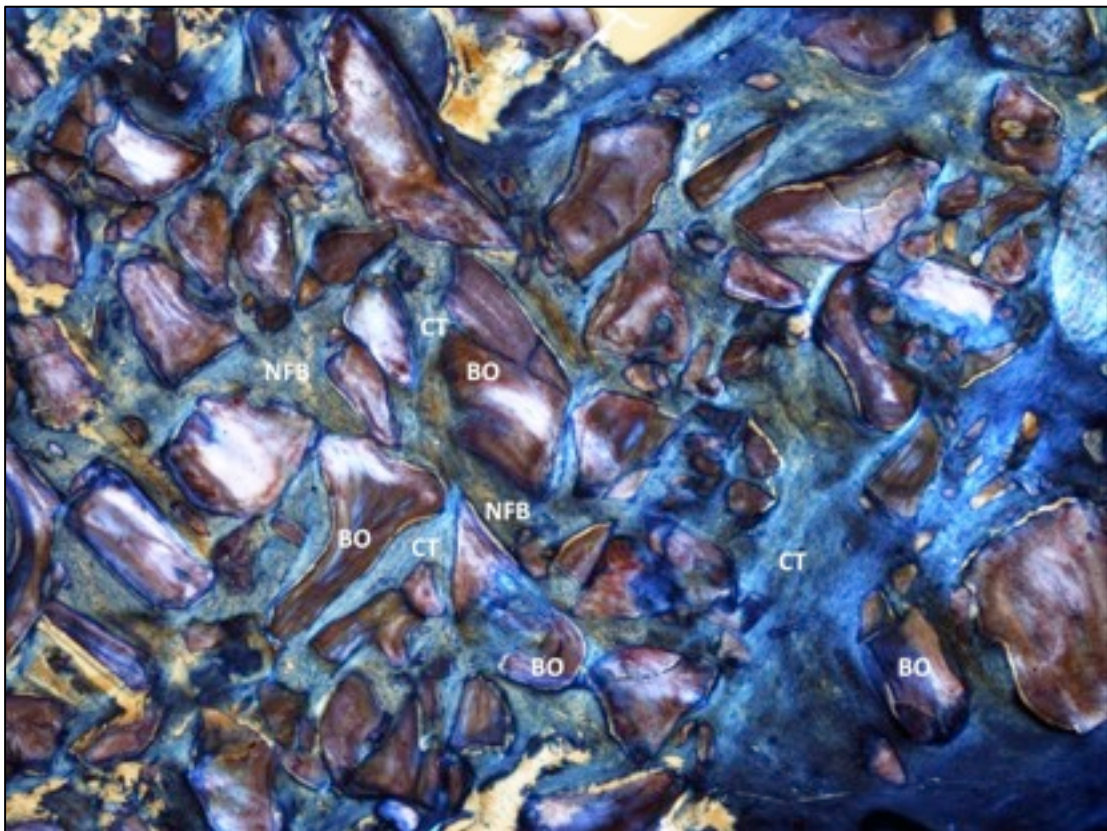


Figure 6: Photomicrograph at polarized light illustrating augmented alveolar socket at 3 months of healing. A great amount of Bio-Oss Collagen particles (BO) was surrounded by highly cellular, fibrous connective tissue (CT) through the entire tissue sample. Minimal amount of newly formed bone (NFB) and no inflammatory infiltrated were detectable in connective tissue and surrounding the bone particles. Original magnification 4x.



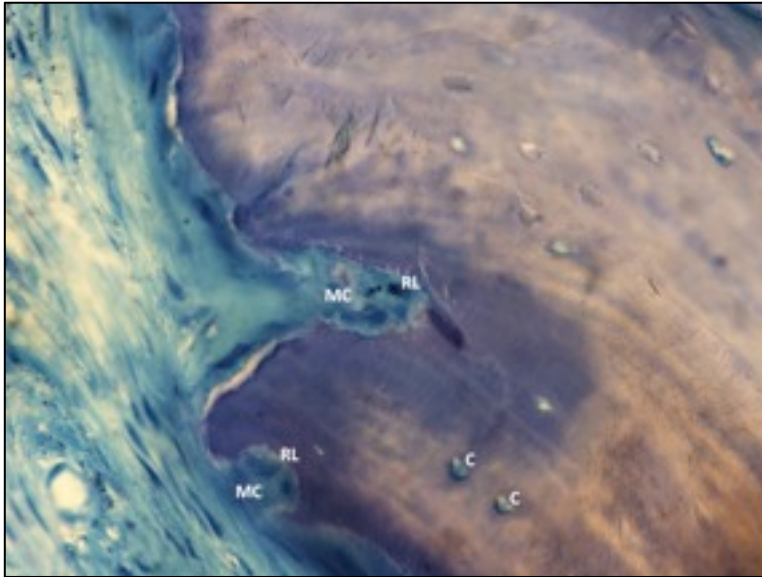


Figure 7: Higher magnification of Figure 6 showing the Bio-Oss Collagen resorption lacunae (RL) and multinucleated cells (MC). The graft particle appeared colonized by cells (C). Original magnification 40x.

At 6 months (Figure 8) a reduced amount of Bio-Oss particles remained in the wound site surrounded by newly formed bone and connective tissue, no ongoing resorptive activity was observed. Implant material is detected laterally, in the apical and coronal side of the sample. The newly mineralized tissue is abundant and composed by lamellar bone and woven bone. Bone marrow spaces among bone trabeculae are wide, highly vascularized, poor in adipocytes. A reduced amount of loose connective tissue highly vascularized is detected. No inflammatory infiltrated is observed. The polarized light shows different stages of tissue mineralization and organization.

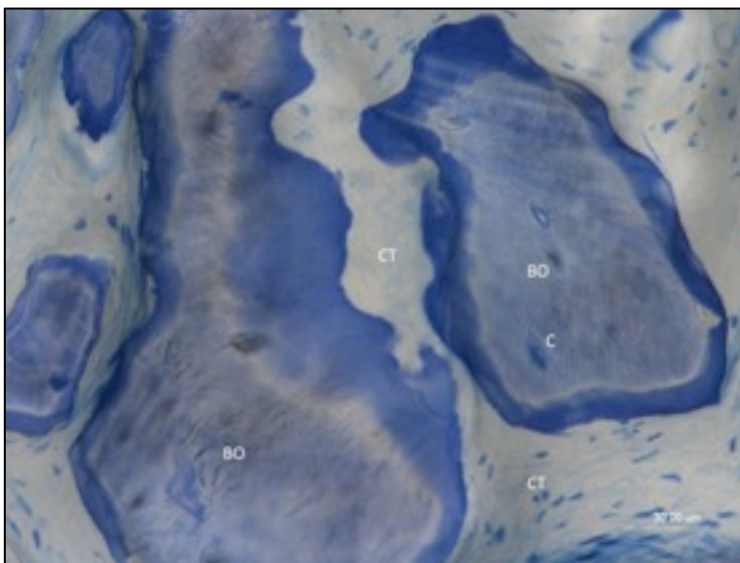


Figure 8: Photomicrograph at 6 month, illustrating Bio-Oss Collagen particles surrounded by cellular, highly vascularized and loose connective tissue (CT). Lacunae of implanted material appeared colonized by host cells (C). No ongoing resorptive activity and inflammatory infiltrated was observed. Original magnification 60x.

At 9 month newly formed bone tissue extends throughout the sample (Figure 9, 10). The mineralized tissue is mainly organized in lamellar shape, and a small amount of woven bone is detectable. Bio-Oss Collagen remnants overall the sample are surrounded by new bone and provisional matrix; no resorption areas are evident. At observation by means of polarized light, implanted material appears surrounded by lamellar bone. Connective tissue extending between bony trabeculae is highly vascularized, loose and slightly cellular. Few marrow spaces are detectable.

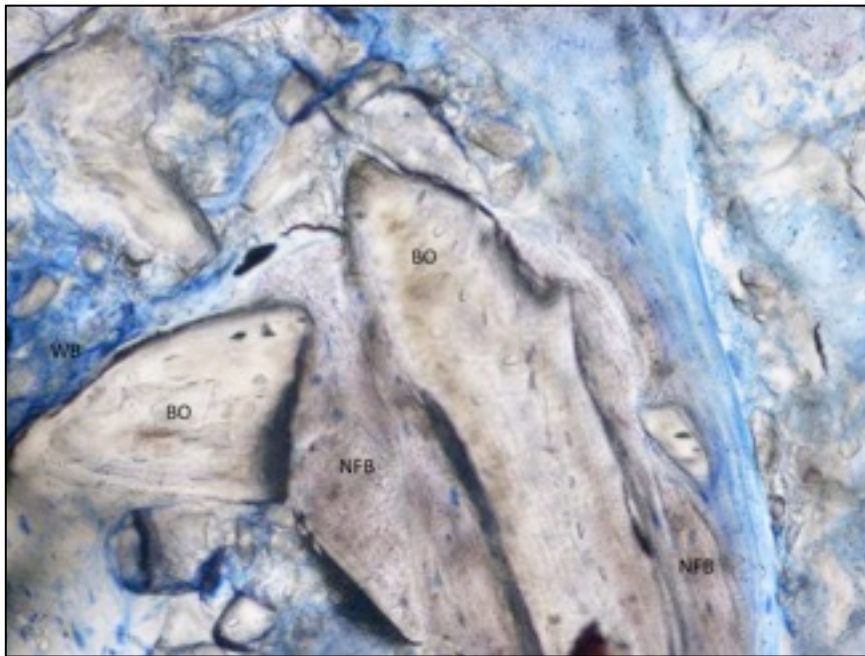


Figure 9: Photomicrograph illustrating newly bone formation at 9 month of healing. Newly formed bone (NFB) tissue extended throughout the sample. The mineralized tissue was mainly organized in lamellar shape, and a small amount of woven bone (WB) was detectable. Bio-Oss Collagen remnants (BO) overall the sample were surrounded by new bone. Original magnification 10x.



Figure 10: Photomicrograph at 9 month showing Bio-Oss particle (BO) in contact with newly formed lamellar bone (NFB). No resorption area was evident. Original magnification 60x.

#### *Histomorphometric results*

At 3 months, mean bone density was 2.77%, residual Bio-Oss Collagen amounted to 43.75%, and connective tissue was 53.48%.

At 6 months, the harvested cylinder was comprised of 44.42% new bone, 12.50% grafted Bio-oss Collagen and 43.08% connective tissue/ marrow.

At 9 months, regenerated bone occupied 47.17% of the total area, Bio-oss Collagen remnants 17.40% and connective tissue/marrow 35.43%.

#### *Alveolar socket filling and importance of buccal wall*

At 3 and 6 months, data on socket depth reduction and on alveolar ridge volume changes showed no significant difference comparing Center, P/A, P/C, P/O measurements between test and control group ( $p > 0.05$ ). To evaluate buccal bone changes, four-wall and BWM defects were separately analyzed.

Four-wall control sites showed an ongoing vertical ridge dimension loss (B/O 3 month: -2.2mm; 6 month: -5.7mm) (Table 2), on the contrary, four-wall test sites preserved the buccal bone height (B/O 3 month: 2.7mm; 6 month: 0.7mm). At the evaluation BWM sites also preserved the buccal bone height (B/O 3 month: 1mm, 6 month: 0.6mm).

*Implant placement and sinus augmentation*

The augmentation procedure was decided for 1 patient in the test group and for 3 patients in the control group (Table 2). For the remaining 5 patients of the test and in the control group, no additional procedure was necessary for implant placement (Table 2). However at the end of the study, dental implants were placed in 4 patients included in the test group and 5 of the control group (Figures 11 a-c, 12 a-c). The remaining patients did not receive dental implant for economic reasons.

	B/O		Center		Sinus augmentation (N)
	Initial Measurements	Baseline/6M	Initial Measurements	Baseline/6M	
<b>Test</b>					
N*	6	6	6	6	1 Needed
Average (min;max)	14.3 (11;20)	0.7 (-5;8) 5.6	19.3 (15;24) 3.3	5.8 (2;9) 2.4	5 Not-Needed
SD	3.3				
<b>Control (4walls)</b>					
N*	3	3	3	3	1 Needed
Average (min;max)	8.3 (6;12) 3.2	-5.7 (-1;-9) 4.2	20.7 (17;23) 3.2	6.7 (4;8) 2.3	2 Not-Needed
SD					
<b>Control (BWM)</b>					
N	5	5	5	5	2 Needed
Average(min;max)	16.4 (13;23)	0.6 (-6;3)	19.8 (12;30)	5 (-1;10)	3 Not-Needed
SD	4	3.9	7.8	4.5	

Table 2: Data on mean changes of alveolar ridge height in test and control groups (BWM and 4 walls). Differences between mean data taken at baseline and 6 months are indicated. Negative data express bone loss and positive data indicate bone gain. (6M: 6 month evaluation, B/O: Buccal-Occusal, N: number of sites, SD: standard deviation, BWM: buccal wall missing, min;max: minimum and maximum value, \*:1 patient did not completed the 6 month measurements and was excluded also from the initial measurements).

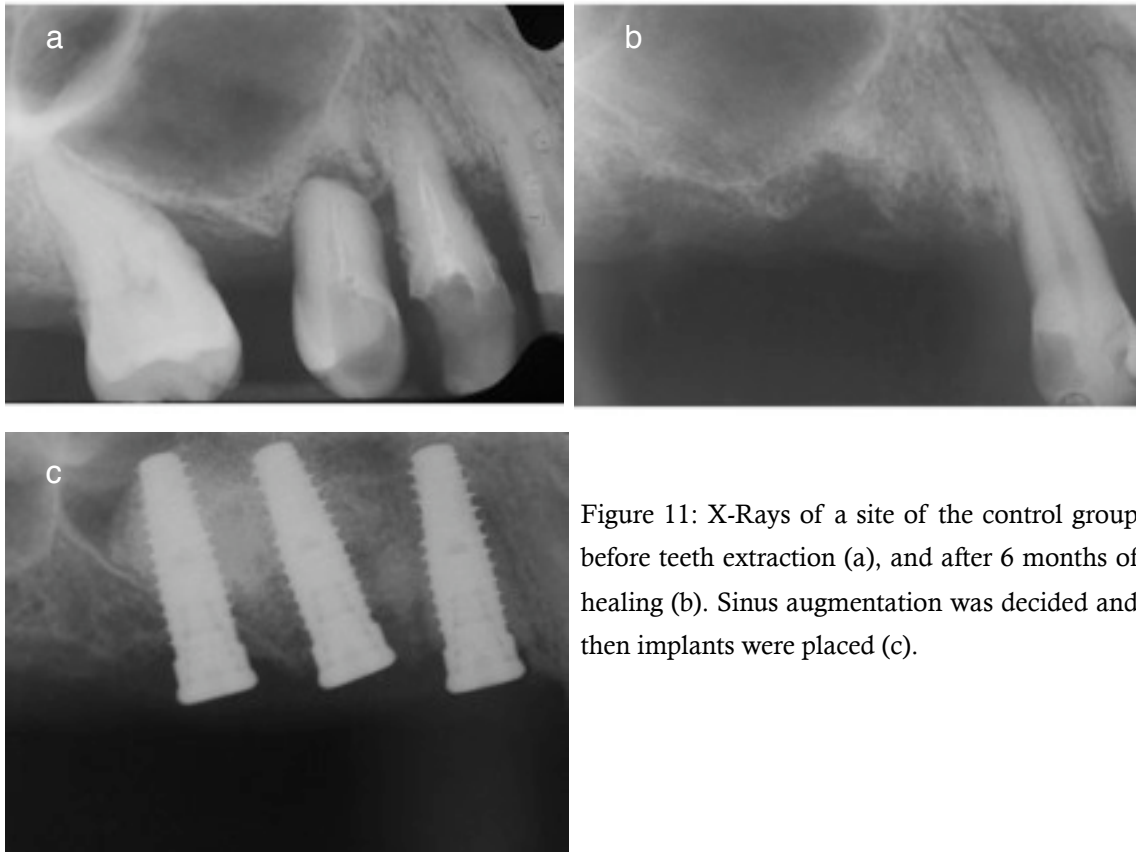


Figure 11: X-Rays of a site of the control group before teeth extraction (a), and after 6 months of healing (b). Sinus augmentation was decided and then implants were placed (c).

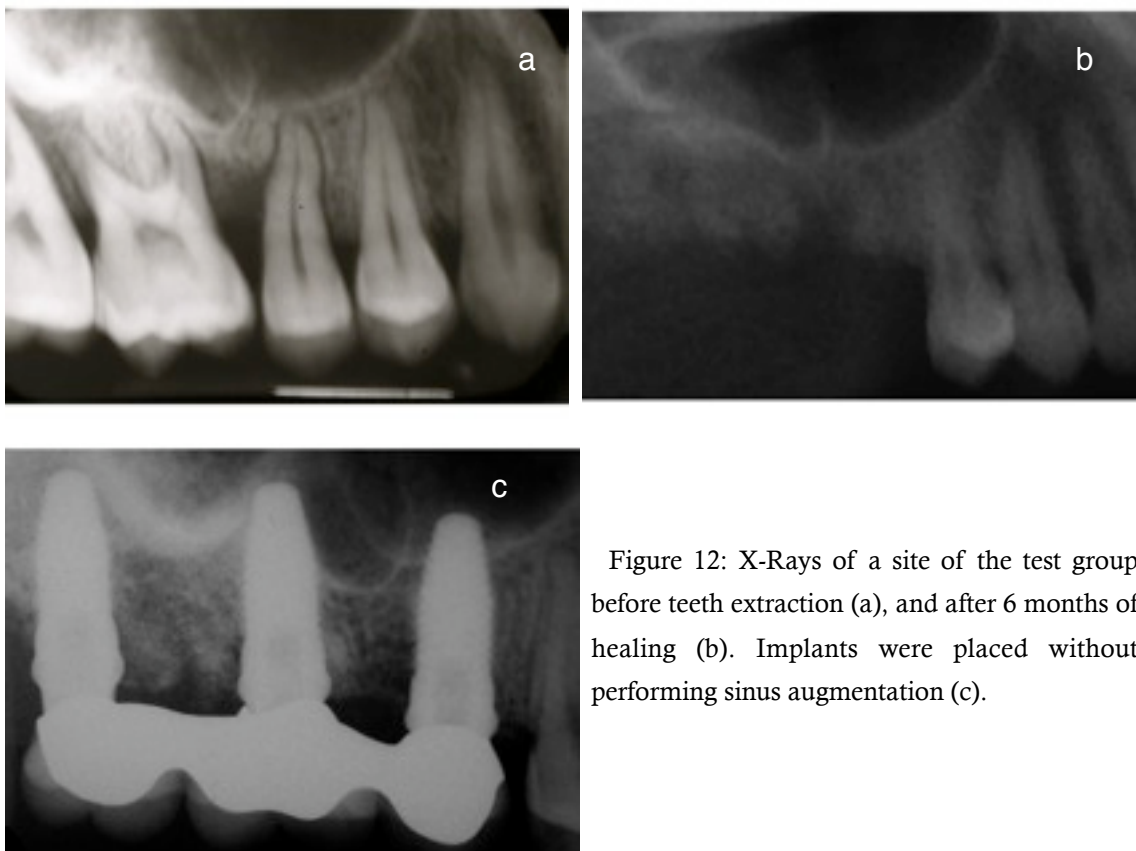


Figure 12: X-Rays of a site of the test group before teeth extraction (a), and after 6 months of healing (b). Implants were placed without performing sinus augmentation (c).

## Study B: materials and methods

This is a split-mouth double blind randomized clinical trial.

In two clinical centers were recruited a total of 14 patients that need the extraction of two contralateral maxillary premolar teeth. All subjects presented the following inclusion criteria:

- Age more than 18 years
- Non existence of systemic disease, hypertension, and metabolic bone disorders (i.e. Diabetes)
- No coagulation disorders
- No intake of drugs inducing gingival hyperplasia (i.e. phenytoin, cyclosporine, nifedipine)
- No current pregnancy, intake of birth control pill, and menopause
- No history of malignancy, radiotherapy, or chemotherapy for malignancy in the past 5 years
- Absence of periapical pathology or suppuration
- Full Mouth Plaque Score and Full Mouth Bleeding Score less than 25%
- No history of periodontal surgery in the experimental site
- No presence of keloid scars.

Informed consent was obtained from all subjects to be entered in the study.

### *Randomization*

Each patient contributed with two experimental sites: one site was assigned randomly to the control group and one to the test group. Furthermore seven patients were randomly assigned to the early endpoint group, and seven to the late endpoint group.

To assign the treatment and the endpoint, Central Registrar designed tables with a balanced random permuted block approach. The treatment and the endpoint for each patient were communicated to the operator immediately after tooth extraction. In order to reduce the chance of unfavourable splits between test, control, early endpoint and late endpoint groups in terms of key prognostic factors, the randomisation process will take into account the following variables: first or second premolar site.

### *Surgical procedure*

At the appointment before surgery, all patients received professional oral hygiene procedures and instructions to eliminate any infective complication. A micro-invasive procedure was used for tooth extraction and if necessary roots were separated with a bur and removed separately (Figure 13). A careful curettage of the socket was performed from the bottom up to the gingival margin with an accurate clean up of the sulcular epithelium. In test sites the alveolar socket was filled with PBBM particles (Bio-Oss Collagen, Geistlich Pharma, Switzerland) and covered with non cross-linked collagen membrane (Bio-Gide, Geistlich Pharma, Switzerland) (Figure 14a). In the control sites the alveolus was only grafted with PBBM particles (Bio-Oss Collagen, Geistlich Pharma, Switzerland) and left uncovered (Figure 14b). In both test and control sites, flaps were drawn up with a Gore-Tex non-resorbable 5-zero suture and let to heal for secondary intention. Patients came for routinely controls every week after surgery for one month (Figure 15). After 5 weeks for the early endpoint group (Figure 16) and after 12 weeks for the late endpoint group, soft tissue samples were harvested in the center of the healing site both from the test and control sites with a punch biopsy.

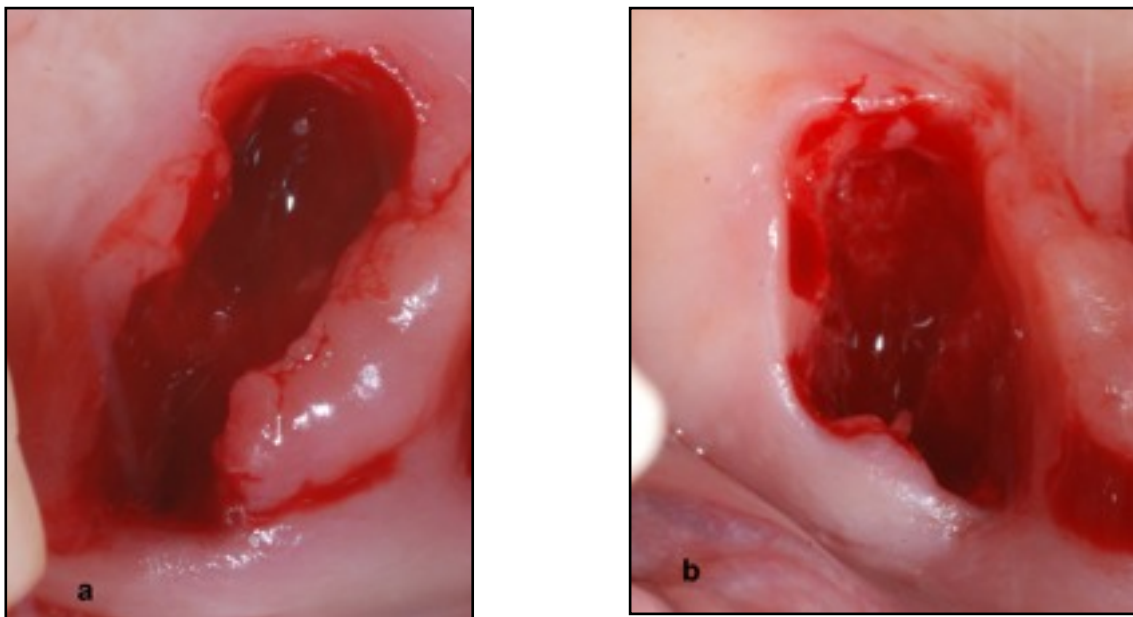


Figure 13: Alveolar sockets of test (a) and control (b) sites immediately after extraction.



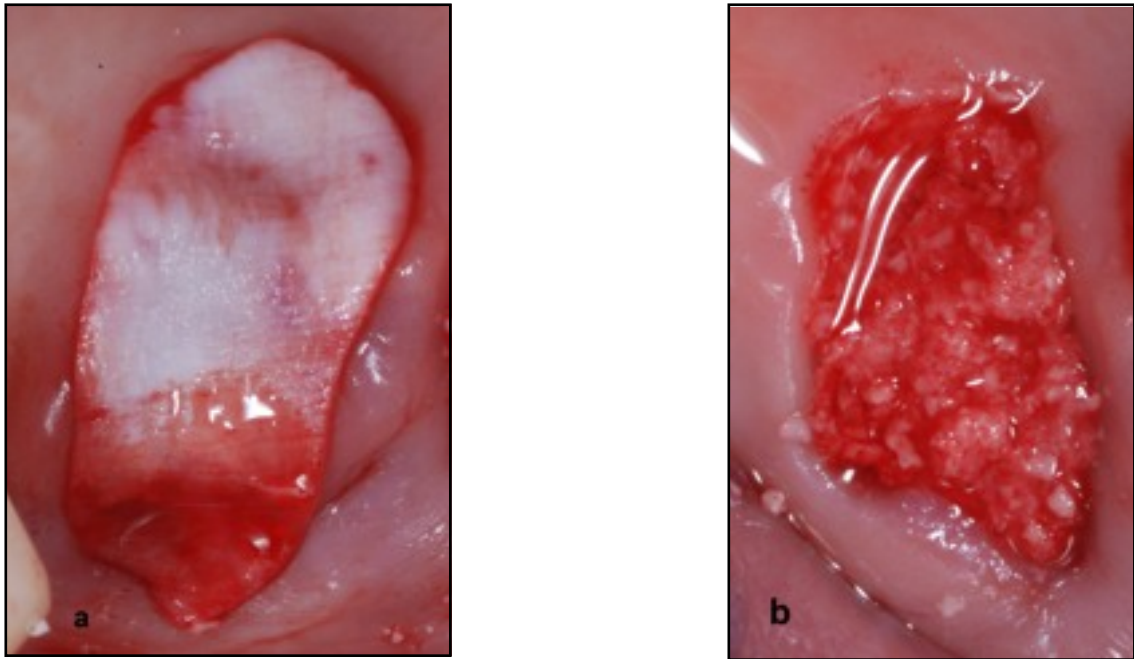


Figure 14: Alveolar sockets of the test sites were grafted with PBBM particles and covered with collagen membrane (a). On the control sites only PBBM particles were grafted (b).

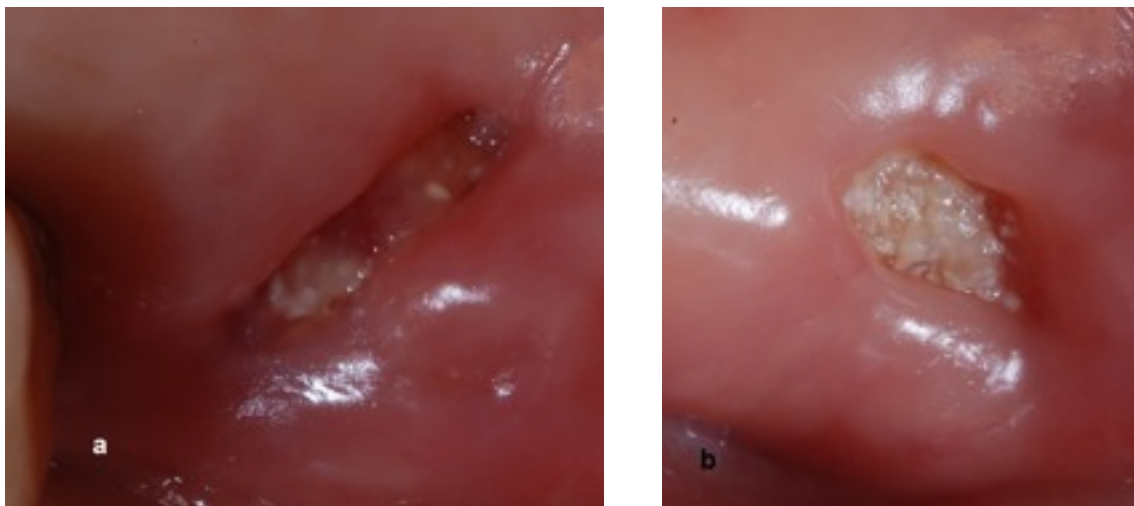


Figure 15: Extraction sites 2 weeks after surgery. In test site (a) a thin layer of soft tissue seem to cover the graft particles. In the control group the filling material seem to be directly exposed to the oral environment.

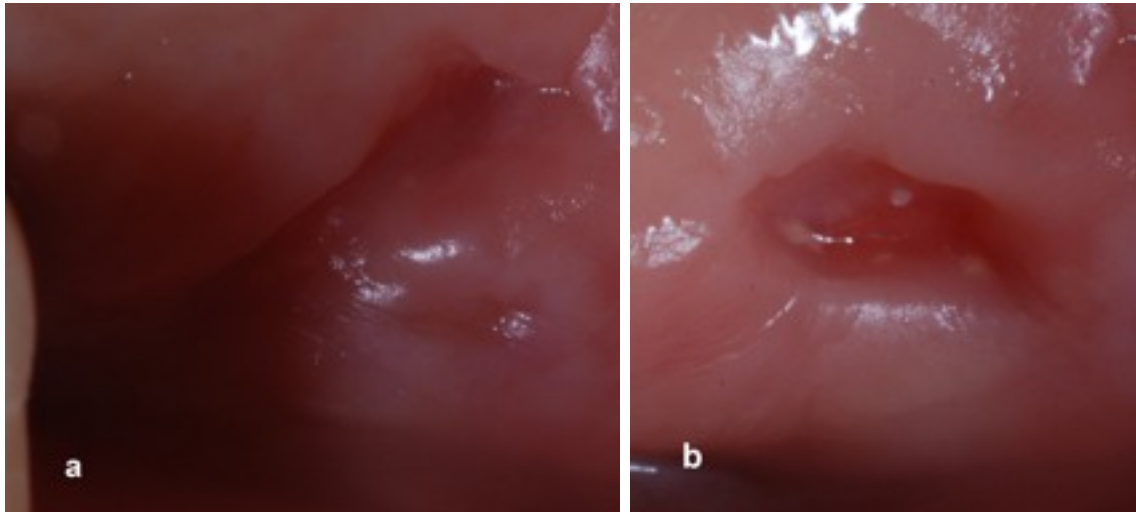


Figure 16: Extraction sites 5 weeks after surgery. Both sites seem to be completely epithelized. Small PBBM particles can be observed trapped within the newly formed soft tissue. a: test site, b: control site.

#### *Specimen processing*

Immediately after harvesting, soft tissue biopsies were immersion fixed in 10% formalin/0.1M phosphate buffer saline (PBS) (pH 7.4) 24 hours at room temperature, then routinely dehydrated in increasing concentrations of ethanol (from 50 to 100%), xylol for 12 hours and then paraffin embedded. Serial 4-5 $\mu$ m buccal-lingual sections were obtained, mounted on 3-Amino-propyl-trietoxi-xilane coated slides and then hydrated in decreasing concentration of xylol and ethanol (from 100 to 70%) and after all immersed in distilled water. To evaluate the tissue morphology four sections for site were stained with Mayer's Haematoxylin (Bio-Optica, Milan, Italy) and Eosin (Bio-Optica, Milan, Italy) according to the standard protocol. To evaluate the collagen content in the connective compartment (Gagliano et al. 2005, Lorencini et al. 2009) four sections for site were stained with Sirius red Picrate (Bio-Optica, Milan, Italy). Sections were incubated at room temperature for 15 minutes in the solution.

*Immunohistochemistry*

Four slides for each sample were incubated with each of the following primary antibody:

- CD20 (pre-eluted, Bio-Optica, Milan) (Figure 17) and CD3 (1:50, Santa Cruz biotech, inc.) (Figure 18) to characterize B and T cells of inflammatory infiltrate;
- CD31 (pre-eluted, Bio-Optica, Milan) to characterize blood vessels (Figure 19).

Sections of human tonsils were used to determine the optimal antibody dilution. Immunohistochemical staining was performed using the polymeric HRP method. For antigen retrieval, slides were treated with protease K eluted in EDTA pH=8 solution and maintained in at 37 °C for 20 min. Slides were incubated for 10 min with H<sub>2</sub>O<sub>2</sub> at 3% to block endogenous peroxidase activity. Slides were then incubated with primary antibody (CD20, CD3, CD31) for 30 min at room temperature. Primary antibody Enhancer (Thermo Fisher Scientific, Fremont, CA, USA) was applied and incubated for 10 min at room temperature. Slides were treated with HRP Polymer (Thermo Fisher Scientific, Fremont, CA, USA) and incubated for 15 min. After incubation with diaminobenzidine tetrahydrochloride (DAB) as substrate/chromogen (“DAB plus chromogen-substrate pack”, Thermo Fisher Scientific, Fremont, CA, USA) for 5 min, the specimens incubated with CD20 and CD31 were counterstained with Mayer’s Haematoxylin and coverslipped. Slides incubated with CD3 antibody were not counterstained. For negative control two sections were incubated in serum.

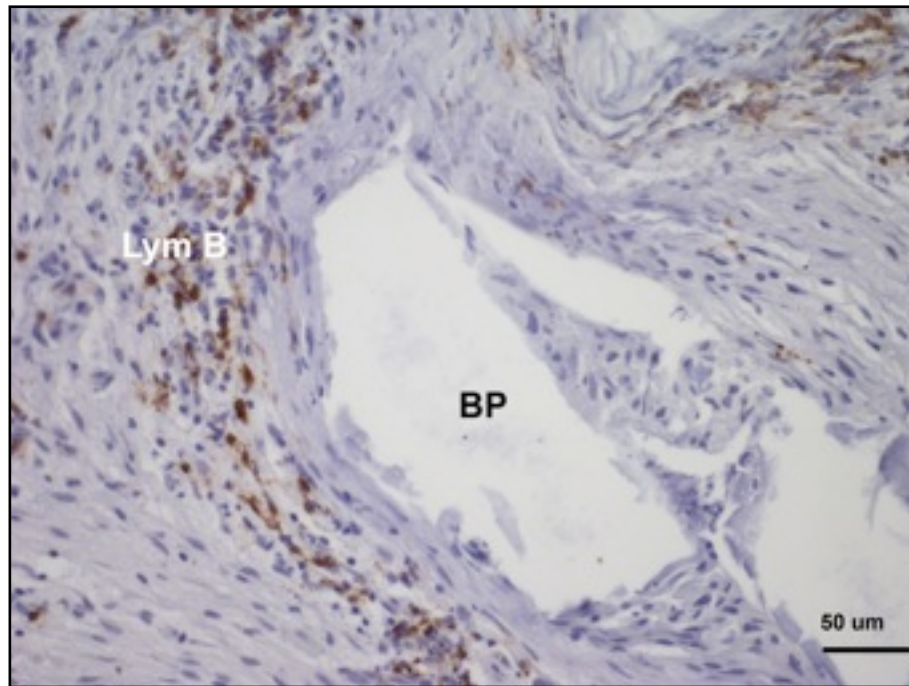


Figure 17: Photomicrograph of test sample harvested 5 weeks after surgery. Tissue immunostained with CD20 for detection of lymphocytes B (Lym B) and counterstained with Mayer's Haematoxylin. BP: bone graft particle. Original magnification 200x.

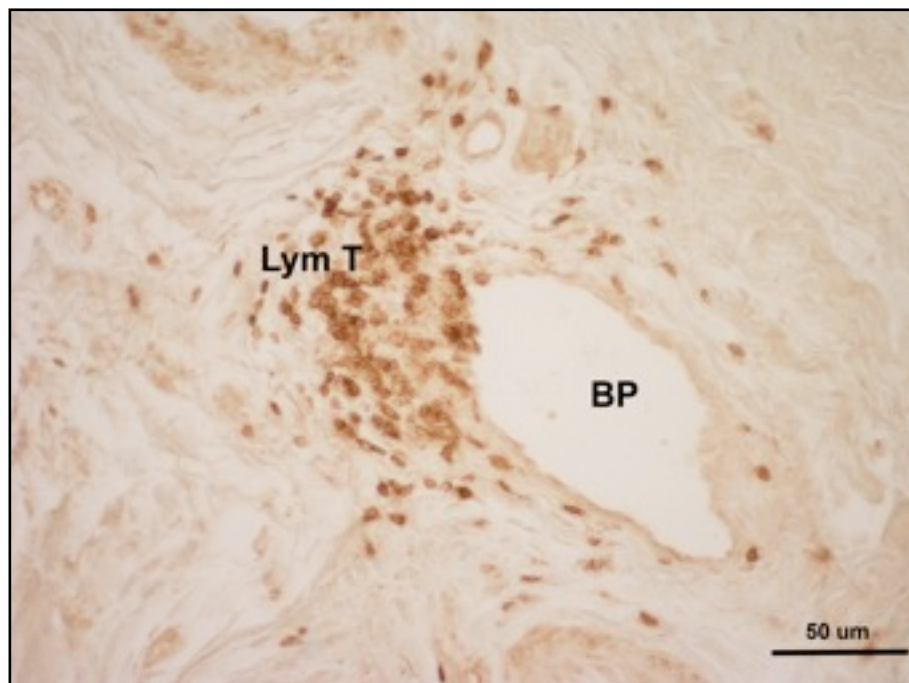


Figure 18: Photomicrograph of a control sample harvested 5 weeks after surgery and immunostained with CD20 for detection of lymphocytes T (Lym T). BP: bone graft particle. Original magnification 200x.

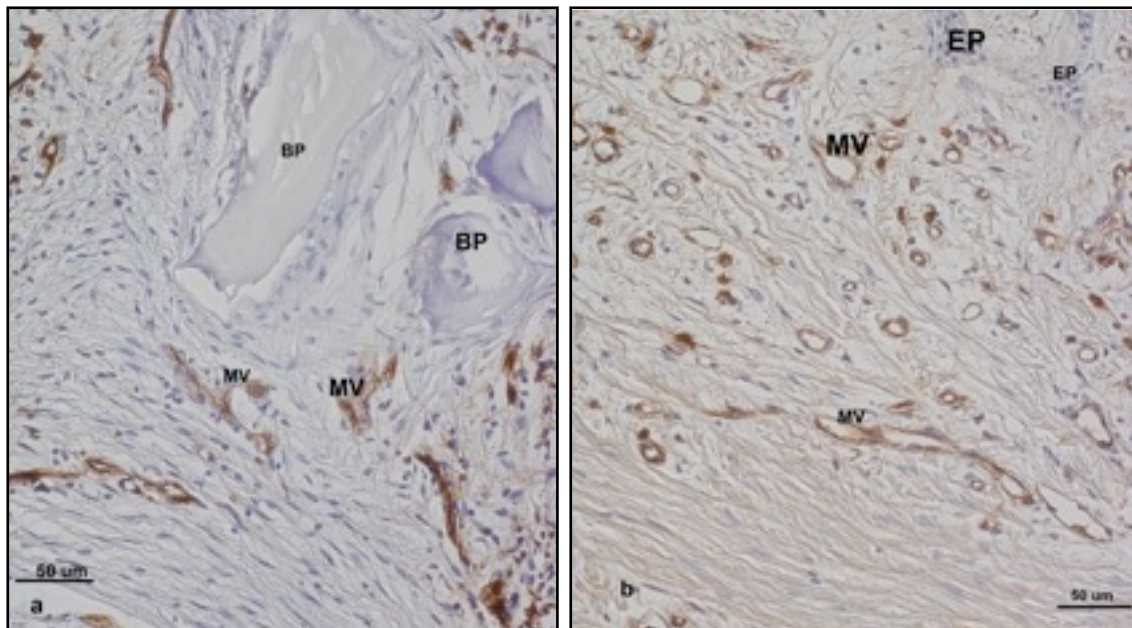


Figure 19: Photomicrographs of test (a) and control (b) samples harvested 5 weeks after surgery and immunostained with CD31 for blood vessels. Control sites presented a higher density of newly formed vessels. BP: bone graft particles, EP: epithelium, MV: microvessels. Original magnification 200x.

#### *Histomorphometrical analysis*

All samples (stained and immunostained) were observed with a Nikon light microscope (Eclipse E600). In sections treated with CD20 and CD3 antibodies, the marked tissue was isolated from the remaining tissue and the content of lymphocytes B (CD20) and T (CD3) was expressed by an area fraction index (Lym B and Lym T). Quantification of variables (CD20, CD3) was performed using 10 fields at total high magnification of 400x (Dellavia et al. 2011). For sections treated with CD31 antibodies, percentage of microvessels in the connective tissue (microvascular density, MVD) was computed using a point-counting technique procedure according to the Delesse formula:  $V_V P_P$ . A lattice comprising 100 test points was superimposed over each histologic section and photographed at a total microscopic magnification of 100X. The tissue underlying each grid intersection was recorded as vessels. The number of hits containing vessels was divided by the total number of intersection fall on the overall connective tissue and thus expressed in percentage values (Canullo et al. 2011). All of the Sirius red-stained sections were analyzed by light microscopy and polarized light, and the images were

captured at a total magnification of 200X and digitized using an image analysis system with specific software (Bio Image Analyzer, ICH, Italy), as described elsewhere (Gagliano et al. 2005). Connective tissue was isolated from the whole gingival section and tissue collagen content was expressed by an area fraction index (AA%), indicating the ratio of the mean Sirius red-stained surface to the connective area of the section.

#### *Statistical analysis*

To evaluate the characteristics of the study population among endpoints groups, Wilcoxon-Mann-Whitney test was used for the age distribution and  $\chi^2$  (chi-squared) test for sex distribution.

Mean, standard deviation and coefficient of variability were calculated for each parameter (Lym B, Lym T, MVD and AA%), separately for test and control groups, for early and late endpoints. For each endpoint, comparisons of these data between test and control groups were computed by Wilcoxon-Mann-Whitney non-parametric test (significant value,  $p < 0.05$ ). Furthermore for each group (test and control), comparisons of all variables between early and late endpoint were calculated by Wilcoxon-Mann-Whitney non-parametric test (significant value,  $p < 0.05$ ).

To test the possible association among variables, the following data series were compared (Pearson's  $r$ ) (strong correlation  $r > 0.70$ , moderate correlation  $0.30 < r < 0.70$ ):

- amount of Lymphocytes B in test and control sites for each endpoint
- amount of Lymphocytes T in test and control sites for each endpoint
- MVD in test and control sites for each endpoint
- AA% in test and control sites for each endpoint

### **Study B: results**

#### *Study population*

Due to personal reasons, two patients of the late endpoint group dropped out. A total of 12 subjects, 7 woman and 5 men (7 of the early endpoint group and 5 of the late endpoint group), were included in the study (Table 3). Patients were all non smoking. To reassume: at 5 weeks after surgery 7 samples of the test group and 7

samples of the control group were harvested and analyzed, and 5 samples of the test and control groups were harvested 12 weeks after surgery. At the statistical analyses, no differences were found between endpoints on sex and mean age ( $p>0.05$ ).

	Early Endpoint	Late Endpoint
Women (n)	4	3
Men (n)	3	2
Mean Age (years)	56.29	53.60

Table 3: distribution of the study population

### *Histological evaluations*

At 5 weeks after surgery samples from the test and control sites did not present significant differences. The epithelium layer was continuous in the central part of the samples, a tissue invagination was observed. The epithelium in correspondence of this central depressed area in few samples appeared not completely keratinized and thinner than the adjacent epithelium (Figure 20, 21). The sub-epithelial connective tissue was overall normal, without signs of tissue necrosis. In few samples small and limited area of increased inflammatory infiltrate was observed adjacent the epithelium. Particles of the bony filling material were detected in both test and control groups and appeared partially degraded, surrounded and in close contact with highly cellular connective tissue (Figure 20, 21). No residuals of collagen membrane were detected in any of the test samples. At 12 weeks after surgery in the sub-epithelial area, the connective tissue was overall normally organized. Small and localized inflammatory areas were still detected. No remaining graft particles were observed (Figure 22). The connective tissue organization was analyzed in slices stained with Sirius-Red (Figure 23, 24). In samples of early endpoint groups the collagen fibers were loose and sparsely organized (Figure 23). The late endpoint sites showed a more mature and organized connective structure, where thick and well organized collagen bundles appeared well oriented (Figure 24). A more evident change in collagen fiber content and organization was observed in test samples.

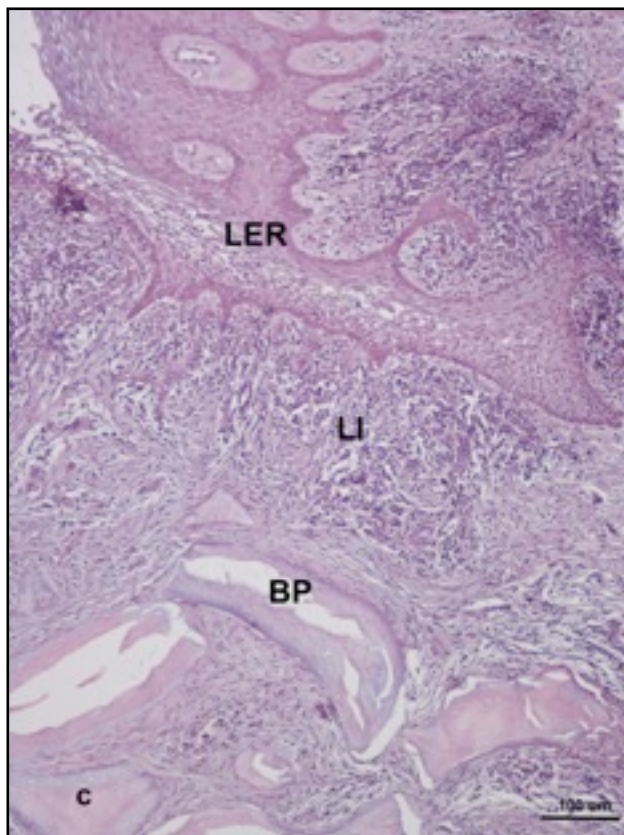
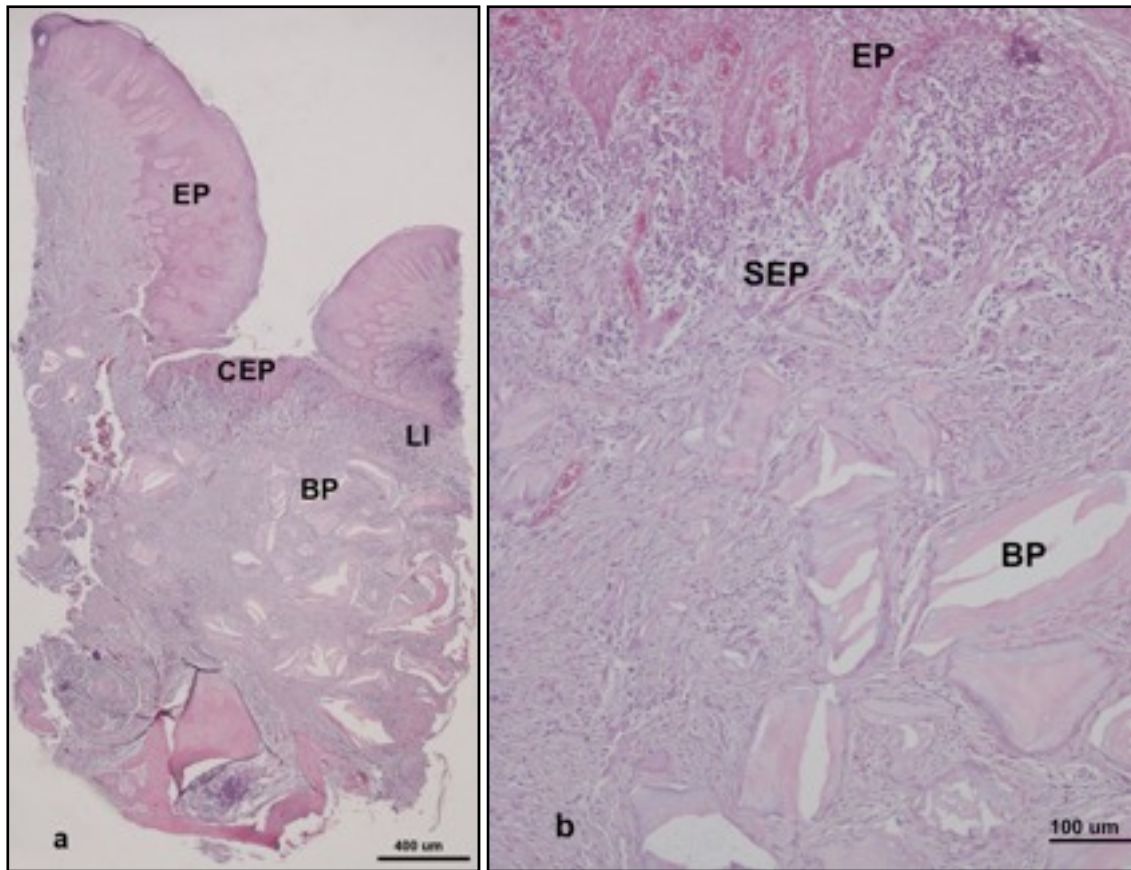


Figure 20: Photomicrograph (a) of test site 5 weeks after treatment and corresponding higher magnifications (b, c). The central part of the defect (CEP) presents a delayed healing status, it results invaginated and thinner than the adjacent epithelium (EP). The sub-epithelial area (SEP) was characterized by a moderate inflammatory infiltrate. A localized inflammatory (LI) zone was also detected surrounding the long epithelial ridge (LER). The LER departs from the point of matching between the central and immature epithelium (CEP), and the more mature adjacent soft tissue (EP). No remnants of collagen membrane results, while bony graft particles (BP) are sparsely distributed within the highly cellular connective tissue. Hematoxylin-Eosin staining. Original magnification 25x (a, c) and 100x (b, d).



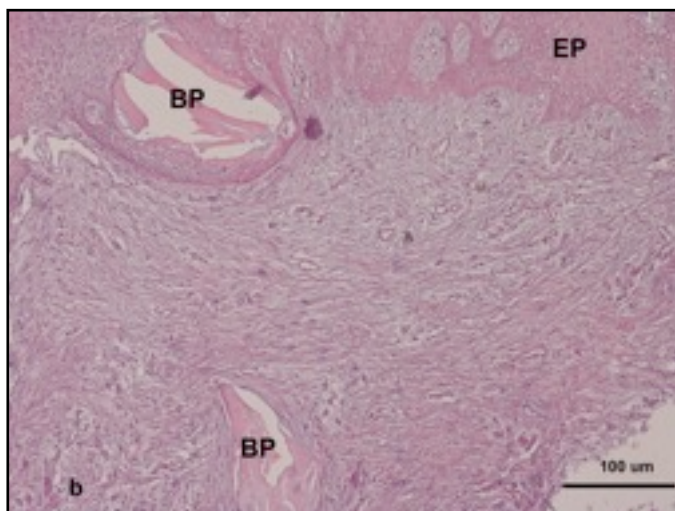


Figure 21: Photomicrograph of test (a) and control sites (c), and the corresponding higher magnifications (b, d) 5 weeks after treatment. Epithelium layer appears intact. In sub-epithelial area (SEA) localized inflammatory (LI) zone was detected. No residual of collagen membrane results, while bony graft particles (BP) are sparsely distributed within the highly cellular connective tissue (HCCT). Hematoxylin-Eosin staining. Original magnification 25x (a, c) and 100x (b, d).

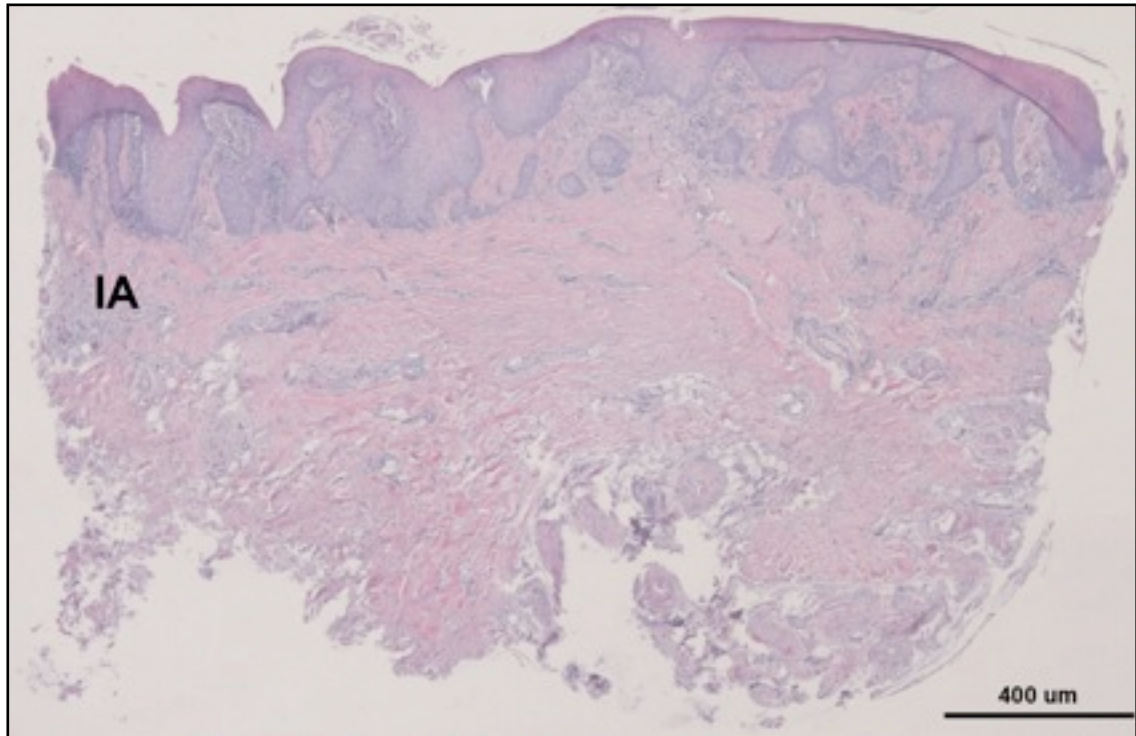


Figure 22: Photomicrograph of test sample 12 weeks after surgery. No remaining graft particles were detected, while a limited inflamed area (IA) was still detected in the sub-epithelial zone. Hematoxylin-Eosin staining. Original magnification 25x.

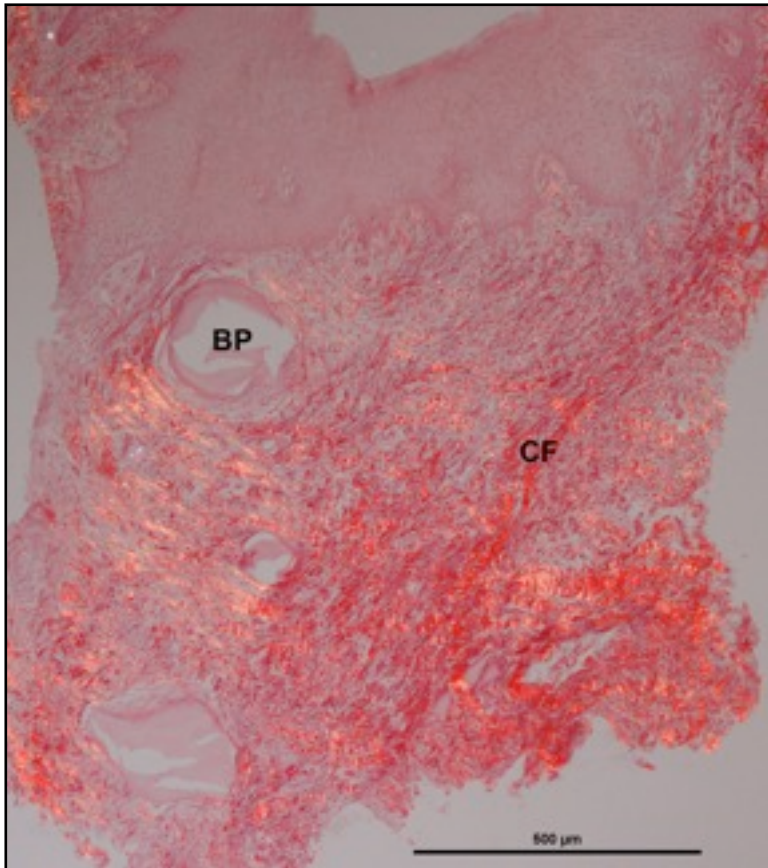


Figure 23: Photomicrograph at polarized light of test site at early endpoint. Sparsely organized collagen fibers (CF) run in the connective tissue. BP: remaining bony particles. Sirius-Red Staining. Original magnification 20x.

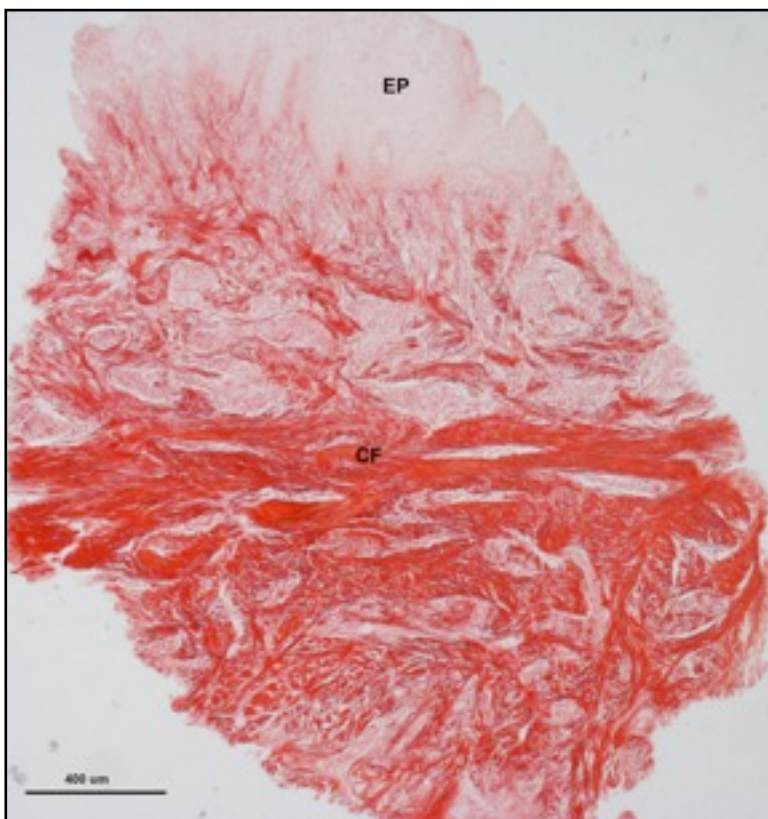


Figure 24: Photomicrograph of test site at late endpoint. Bundles of collagen fibers (CF) run parallel to the epithelium (EP), while small fibers are distributed orthogonally. Sirius-Red Staining. Original magnification 25x.

*Histomorphometric results*

To evaluate the influence of the membrane on soft tissue healing, the differences on micro-vascular density, collagen content and inflammatory cells between tissue samples harvested in sites treated with or without membrane were evaluated.

At 5 weeks (Table 4) the vascularization of tissue in samples sites treated with the graft+ resorbable membrane was significantly lower (MVD= 6.18) than in samples harvested from sites treated only with the filling material (MVD= 9.44) (Wilcoxon Matched Pair-Signed Rank,  $p < 0.05$ ). At this endpoint, data on Lymphocytes T and B, and collagen content showed no significant differences between test and control group. Data on Pearson's correlation of samples of the early endpoint group are indicated in table 4.

At 12 weeks (Table 5) no significant differences between test and control group were found for any of the considered parameter. Data on Pearson's correlation of samples of the late endpoint group are indicated in table 5.

To evaluate the maturation process of the soft tissue, the alterations of vascularization, inflammation and collagen content between the two endpoints were observed (Table 6).

Data from control groups seem to reveal slight increase in tissue inflammation at 12 weeks after surgery, otherwise data from test group show a reduction of both Lymphocytes T and B. The statistical analysis between early and late endpoints was performed comparing for each group (test and control) the data on Lym T and B separately, and also combining the inflammatory content (Lym T+B). No statistically significant difference was found in any of these computations. Data on MVD and collagen content resulted increased at 12 weeks after surgery in both groups, however only data on AA% in test group increased significantly.

	<b>MVD (CD31)</b>	<b>Lym B (CD20)</b>	<b>Lym T (CD3)</b>	<b>AA% (RS)</b>
<b>Test Group EarlyEp (5w)</b>	6.18	1.91	2.58	33.85
<b>Cntr Group EarlyEp (5w)</b>	9.44	1.20	1.72	39.61
Wilcoxon Mann Whitney signed rank	P<0.05	N.S.	N.S.	N.S.
Pearson's (r)	0.72	0.91	0.92	0.37

Table 4: Data on Microvascular density (MVD), content of Lymphocytes B (Lym B), T (Lym T) and collagen (AA%) computed in the test and control groups 5 weeks (5w) after surgery (EarlyEndpoint). The paired statistic and the Pearson's correlation was calculated for each parameter. N.S.: not significant.

	<b>MVD (CD31)</b>	<b>Lym B (CD20)</b>	<b>Lym T (CD3)</b>	<b>AA% (RS)</b>
<b>Test Group LateEp (12w)</b>	9.20	1.01	1.46	51.49
<b>Cntr Group LateEp (12w)</b>	10.26	1.38	3.30	48.04
Wilcoxon Mann Whitney signed rank	N.S.	N.S.	N.S.	N.S.
Pearson's (r)	0.18	0.94	0.85	0.69

Table 5: Data on Microvascular density (MVD), content of Lymphocytes B (Lym B), T (Lym T) and collagen (AA%) computed in the test and control groups 12 weeks (12w) after surgery (EarlyEndpoint). The paired statistic and the Pearson's correlation was calculated for each parameter. N.S.: not significant.

	<b>Wilcoxon Mann Whitney Sum of Rank</b>
MVD test: early vs late	0.07
MVD cntr: early vs late	0.51
Lym B test: early vs late	0.93
Lym B cntr: early vs late	0.8
Lym T test: early vs late	0.62
Lym T cntr: early vs late	0.12
AA% test: early vs late	0.01*
AA% cntr: early vs late	0.29

Table 6: Comparison between early and late endpoints for: microvascular density (MVD), Lymphocytes B and T, collagen content (AA%). Each parameter was analyzed separately for test and control groups. A statistics test for unpaired group was performed. \* :significant ( $p < 0.05$ )

## Discussion

In the first section of this research (study A), alveolar sockets were treated with PBBM filling material and covered with resorbable membrane. At different endpoints, the healing of the hard tissue and the integration of the biomaterial were described histomorphometrically; furthermore the volumetric reduction of the hard tissue and the need of further regenerative procedures were clinically investigated. Afterward the second section (study B) focused on the soft tissue healing and the role of resorbable membrane on regeneration and maturation of connective tissue.

Data from study A showed 3 month after augmentation a great amount of implanted material on remodeling phase, imbedded in provisional matrix and coated to osteoclast-like cells. No new bone surrounded the remnant particles but areas of dense cellular connective tissue in multiple mineralization phases were observed. These findings are in agreement with previous studies. Cardaropoli et al. (2005) evaluated in dog model the healing sites at 3 month, corresponding approximately at 18 weeks in humans (Giannobile et al. 1994). Implanted material

detected throughout the section occupied a substantial portion of tissue that filled the defect. In central region bovine bone particles were surrounded by connective tissue, while in lateral areas they were embedded in newly formed woven bone. Previous animal studies also showed multinucleated cells coating and replacing the xenograft material with new host bone (Berglundh et al. 1997, Araújo et al. 2009). Heberer et al. (2008) displayed the Bio-Oss Collagen augmented extraction socket at 6 weeks in humans and the newly formed bone ongoing from the apical and lateral areas. In coronal-central areas particles were imbedded in provisional matrix. Becker et al. (1998) showed the failure of bovine bone particles on promoting bone regeneration in human alveolar socket. Graft particles embedded in dense connective tissue was detected at 3 and 4 month of healing. At histomorphometric analysis, a great amount of Bio-Oss Collagen (43.75%) and a limited amount of newly formed bone (2.77%) were detected in the present study. To the contrary, previous animal (Araujo et al. 2008) and human (Heberer et al. 2008) studies evaluating early healing phases found a limited amount of implanted material (18.9% and 11% respectively) and a greater percentage of mineralized bone (14.7% and 28% respectively). In all studies, connective tissue represented a substantial portion of healing tissue. In the animal study, data regarding unfilled site were use as control; a high amount (47.8%) of newly formed bone was observed immersed in provisional matrix (49.1%).

In the present study, histological evaluation of unfilled defects was not performed. Previous human studies described bone formation process arising from the residual bone walls at the apical and lateral defect sides (Amler 1969). In a dog study (Cardaropoli et al. 2003) bone healing dynamics have been well described at subsequent timepoints. At 60 and 90 days, corresponding at 3 to 4-5 month of human healing, the specimens showed marrow spaces and woven bone beginning to be replaced by lamellar bone.

At 6 month a small amount of Bio-Oss particles remained at lateral areas of the sample, while the center was occupied by marrow spaces, newly formed lamellar and woven bone organized in trabeculae. Bovine bone particles are mainly surrounded by loose connective tissue, and only a limited amount is in contact with woven bone. This finding is in agreement with previous studies. Carmagnola et al. 2003 harvested tissue samples at 6 months of healing from alveolar sockets

augmented with Bio-Oss and covered by means of collagen membranes. The authors observed that graft particles were mainly included in connective tissue, and only a small amount was surrounded by newly formed woven. At the same timepoint Molly et al. (2008), in a study on alveolar socket preservation, observed provisional matrix (osteoid tissue) and limited newly woven bone lining Bio-Oss particles. The same study showed the healing process in non-grafted controls. Newly formed bone tissue reached almost the complete maturity displaying osteocytes and Heversian systems. Histomorphometric evaluations at 6 month showed data in agreement with previous human studies (Carmagnola et al. 2003) (Molly et al. 2008) (Cardaropoli et al. 2005). Newly formed bone density was increased to 44.42%, similarly to Carmagnola et al. (2003) (34.4%) and Cardaropoli et al. (2005) (47%). Bio-Oss Collagen was partially resorbed and only 12.5% was detected. Previous studies (Carmagnola et al. 2003) (Molly et al. 2008) (Cardaropoli et al. 2005) (Norton et al. 2003) reported a remaining particles percentage varying between 20.2% and 27%. Evaluations conducted on control unfilled sites (Cardaropoli et al. 2005) showed 39% of newly formed bone and 61 % of connective tissue and bone marrow.

At 9 month histological evaluation showed a high amount of lamellar bone in direct contact with graft particles; small amount of connective tissue was evident. Artzi et al. (2000) showed the healing at 9 month of human extraction sockets filled with Bio-Oss and covered with a pediculated split palatal flap. They also displayed in samples a great graft particles amount and mostly lamellar bone formation. In both studies the newly formed bone represented about 47% of the entire tissue. However in the present study the Bio-Oss Collagen percentage was 17.4 and the previous study represented about 35.1% of the samples. Histomorphometric data on control unfilled sites were nor available.

In a dog study the alveolar socket healing dynamics (Cardaropoli et al. 2003) were described at 120 and 180 days, corresponding at about 6 to 9 month of human healing. Mature bone marrow spaces, containing adipocytes and surrounded by trabeculae of lamellar bone were observed.

Findings of the present study showed a delayed bone healing process. After tooth extraction the blood clot fills the alveolar defect, starting the inflammatory and subsequently the producing, remodeling and modeling processes. In augmented



defect the blood clot fills only the spaces between the implanted material, thus diminishing cellular and molecular amount involved in healing and regeneration process. Moreover implanted biomaterial may elicit a foreign body reaction that is observable in multinucleated cells coating the Bio-Oss Collagen surface (Araújo et al. 2009). These events may result in a delayed bone formation process.

On the other side, as the clinical results of this study showed, the implanted material stabilizes the wound and maintains the form of the edentulous ridge. This implies easier implant placement procedures, more successful prosthetic restorations and esthetics (Nevins et al. 2006). In the present study dental implants were successfully placed in areas where bone core had been retrieved, showing that bone tissue features were adapted for implant placement.

The second part of this research was designed to evaluate the possible interference of the membrane on soft tissue healing.

At the histological observation features of the epithelium in test and control groups at early and late endpoints were similar. This indicates a functional epithelial reconstitution in defects covered with collagen membrane. The application of the collagen membrane for treatment of open skin wounds was proposed in an animal study that assessed the success of membranes for reepithelization (Wehrhan et al. 2010). Long epithelial ridges were observed in few samples of the present study. Previous human histological study (Tal et al. 2008) evaluated soft tissue healing of sites treated with guided bone regeneration. Authors reported these epithelial formations in sites where the collagen membrane exposition occurred and supposed a growth of the epithelium below the membrane (Tal et al. 2008).

An essential aspect of the wound healing is the angiogenesis that delivers nutrients, cytokines and inflammatory cells. Immediately after injury, angiogenic factors (i.e. FGF-2, VEGF...) are released to induce the proliferation, migration and differentiation of endothelial cells in to capillary tubes (Morelli et al. 2011). During the inflammatory phase of the wound healing, the microvascular density increases in the healing tissue driven by the peak of these factors. In the remodelling and maturation phases the angiogenetic cytokines discontinue and the capillary tubes consequently regress (Nissen et al. 1998).

In the present study the soft tissue harvested at the early endpoint from sites treated with resorbable membrane showed a significant lower microvascular density than tissue from control sites. However, at the late endpoint MVD increased in both groups and no difference between test and control sites were detected. Clinical studies described the mucosa features of sites treated with resorbable membrane (Kirkland et al. 2000, Iasella et al. 2003). Kirkland et al. (2000) evaluated the hard and soft tissue dimensional changes in atrophic alveolar sites, edentulous from at least 6 months and treated with graft material and resorbable membrane. The membranes were completely covered with the flap and a primary intention closure was obtained. The authors reported a significant change in soft tissue thickness at 12 months after augmentation procedure and hypothesized that the membrane may interfere with the flap vascularity. Iasella et al. (2003) described the hard and soft tissue healing of post extractive sites left untreated or treated with bone substituted and collagen membrane. The experimental sites were only partially covered with the flap and sutured. Compared to the baseline measurements, at 4 to 6 months after surgery the authors reported the increase of soft tissue thickness in sites left untreated. Otherwise in sites where the membrane was placed to protect the filling material, the mucosa after healing got thinner than at baseline (Iasella et al. 2003). As Kirkland et al. (2000), in this study (Iasella et al. 2003) was hypothesized that membrane and graft reduce the vascular support of the healing tissue and impair the tissue regrowth. Results of the present histological study confirm the interference that the collagen membrane exerts on the angiogenesis process during the initial wound healing. Pre-clinical studies seem to contrast with these findings (Wehrhan et al. 2010, Schwarz et al. 2006). In a study on pigs, Wehrhan et al. (2010) compared the suitability of a bovine collagenous membrane as dermal substitute using an open skin wound model. The authors (Wehrhan et al. 2010) observed a temporarily delayed vascularization in sites left to heal spontaneously (free granulation tissue) compare to sites treated with membrane or split-thickness autogenous graft. Further studies investigated in rats the biodegradation of different resorbable membranes implanted subcutaneously and closed for primary intention (Patino et al. 2003, Schwarz et al. 2006). Non Cross-linked collagen membrane (Bio-Gide) showed the homogeneous and almost complete transmembranous formation of blood vessels 2 weeks after implantation. The structure of Bio-Gide

appeared most suitable for a premature angiogenesis. Furthermore blood vessels formation seemed to be proportional to the biodegradation of the membrane body (Schwarz et al. 2006).

It would be emphasized that these studies (Patino et al. 2003, Schwarz et al. 2006) were designed to assess the growth of the newly formed tissue within the collagenous structure. For this purpose the authors used a model where the membrane was completely enclosed in the subcutaneous tissue. Otherwise the intention of present study was to observe the features of the mucosa that grew up and overlaid the membrane.

Wehrhan et al. (2010) proposed an open skin wound model that may appear similar to the model proposed in this study. However in the present research the collagen membrane was left exposed to the oral cavity. Animal studies (Tal et al. 1992, ...) reported the complete degradation of resorbable membranes within few days when exposed to the oral environment. Most properties of the collagen device should be attributed to the 3-dimensional structure. The rapid disintegration operated by oral collagenase and bacterial colonization may jeopardize the beneficial effects of the membrane on vascularization and tissue ingrowth. Thus their results should be considered as a proof of principle of the interaction between the living tissue of the animal and the collagen structure, but different behavior may be expected in clinical application.

The inflammatory reaction to different collagen membranes was also investigated in animal models (Patino et al. 2003). A study on cellular response to non cross-linked collagen membrane (Bio-Gide) reported only a slight transient and local augmentation in tissue macrophages as a result of a foreign body reaction. No increased levels of lymphocytes T and B were observed when compared to the control sites (saline solution). In the present study levels on monocytes in the test group was slightly increased at 5 weeks after surgery and decreased at the late endpoint, but no difference was found with control sites. At the analysis, for each patient the amount of lymphocytes B and T in test and control sites resulted closely correlated. Despite the small number of samples analyzed in this research, this data seem to indicate that the inflammatory levels are not related to the membrane placement, but to individual factors; the biocompatibility of the non cross-linked membrane seems to be confirmed.

A further important results of the present study concerns the collagen content. Human histological studies on collagen fibers and periodontal healthy (Seguier et al. 2000, Ejeil et al. 2003) reported a close relation between gingival inflammation and collagen fibers remodelling. Metalloproteinases, inflammatory infiltrating cells, bacteria and resident cells are activated by inflammatory events and induce the connective tissue degradation. In these studies the area fraction AA% occupied by collagen bundles in healthy and mature gingival tissue was 50% (Ejeil et al. 2003) and 60% (Seguier et al. 2000). In the present study, the AA% increased at the late endpoint to values (51.49% for the test group and 48.04% for the control group) observed in healthy gingival samples. Particularly, in the test group this augmentation was more relevant than in the control group.

This study confirms that also in alveolar socket augmented sites, the collagen content and fiber organization of the soft connective tissue is related to the tissue maturation. During the first healing phases after injury, the tissue presented a reduced amount of collagen content, the fibers were loose and not well organized in bundles. In the maturation phase the collagen matrix was remodelled in a more organized and dense fibrous structure. Soft tissue samples of the test sites showed an initial delayed healing process with a lower microvascular density and collagen content, while subsequently they seem to gain the same maturation level than tissue samples of the control group. The sites treated with membrane were characterized by a strong augmentation of collagen fibers and microvascular density.

Despite the limited number of patients, data from this study seem to confirm that the collagen membrane induces a modification of the normal wound healing. It would be interesting to discuss further if the membrane degradation elicits a foreign body reaction thus delaying the initial phases of the soft tissue healing or the barrier effect of the membrane block initially the cellular migration from the underling bone tissue. Since the rapid membrane degradation, studies should be designed to investigate the histological events on the treated site, few hours and days after augmentation.

## Conclusion

Management of the tissue reconstruction is one of the coolest topics in regenerative medicine, thus a great variety of biomaterials and biologics are constantly proposed to control and guide the cellular migration and activity.

Alveolar socket extraction may be considered a useful human model to assess the interaction of new medical formulations (biomaterials or biologics) with the bone tissue and to evaluate their efficacy on bone regeneration. Advantages of this model are: i) the possibility to harvest easily tissue samples at the implant placement appointment, ii) the minimally invasive surgical procedure that is needed for augmentation, iii) the large amount of histological, clinical and pre-clinical studies published on spontaneous or guided alveolar socket healing. The post-extractive defect has been treated with several regenerative systems: membranes alone (Pinho et al. 2006, Neiva et al. 2011), grafts (i.e. Autograft, xenograft, synthetic bio substitutes) covered with membranes (Artzi et al. 2000, Carmagnola et al. 2003), grafts mixed to growth factors (Becker et al. 1998) and bone reparative cells (RBCs) produced from bone marrow aspirates and then carried in the experimental site by means of gelatin sponges (Kaigler et al. 2010).

In dentistry, bone, bone substitutes and membrane are the most largely used materials for periodontal and guided bone regeneration. Several studies confirmed the clinical efficacy and the utility of these products. However histological findings demonstrated that these grafts tend to modify the physiologic healing processes, eliciting foreign body reaction and delaying the regeneration process. The tendency in reconstructive medicine is to improve the intrinsic regenerative potential and to recapitulate the embryogenetic mechanisms of tissue formation.

At this purpose new micro-surgical techniques for blood clot protection and stabilization were proposed with or without filling materials (Cortellini et al. 2011, Trombelli et al. 2012). Furthermore the research is oriented on the development of innovative delivery systems for topical release of growth factors as cells therapy, gene transfer or innovative scaffolding matrices (Ramseier et al. 2012, Pagni et al. 2012).

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