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# Deproteinized bovine bone graft remodeling pattern in alveolar socket.

## Histologic and immunohistological expression evaluation.

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**Research**

**Abstract**

Deproteinized bovine bone (DBB) is a bone graft highly used in dentistry in bone regeneration and alveolar socket preservation techniques. Although there is a scientific consensus on the clinical benefit of this biomaterial, and several in vitro studies described its biologic effect on osteoblasts, in vivo analyses investigating its effect on bone dynamics on human are lacking. For this purpose 20 patients needing tooth extraction and implant placement were selected and randomized in test (alveolar socket preservation with DBB) and control (spontaneous healing). Bone specimens were collected during tooth extraction (T0) and, at 5 months, during implant placement (T1). The collected samples were processed for histologic and immunohistological analyses to reveal the presence of positive (BMP-2, BMP-7, ALP) and negative (Il-6, TNF-α) markers of bone remodeling. The sections were then micro-photographed, quantification was done and statistical analyses were performed to compare T0 and T1 in both groups and T1 test group versus T1 control group. The obtained results showed higher expression of BMP-2, BMP-7 and IL-6 at T1 in both groups ($p < 0.05$), lower expression of ALP in both ($p < 0.05$) at T1 and higher expression of TNF-α only in test group ($p < 0.05$) while in control group it remained stable during time. When comparing T1 markers expression in control and test groups, a higher expression of BMP-2 ($p < 0.05$) and lower expression of TNF-α ($p < 0.05$) were found in the first one. These results are in concordance with the previous in vitro studies and show that DBB is able to maintain bone remodeling in active phases. As a matter of fact at 5 months a higher expression of the positive markers (BMP-2, BMP-7) was noticed compared to T0, but the presence of DBB resulted in lower expression of BMP-2 and higher expression of inflammatory factor (TNF-α) when comparing to spontaneous healing at the time of the implant placement.

For these reasons the use of DBB is suggested when clinical needs lead to a precise indication of alveolar socket preservation with biomaterial, while, if not clinically necessary, spontaneous healing is indicated because it shows more biological positive effects.

**Introduction**

**1.1     Bone Morphology**

Bone is a specialized connective tissue and provides different functions for the entire body. It protects inner structure from external injuries and it gives function and retention to tooth. Bone may be defined, from a morphological point of view, as *Alveolar Bone, Cortical Bone* and *Woven Bone*. Alveolar bone origins from dental follicle and cooperates with periodontal ligament and cementum in providing tooth attachment system. Alveolar bone is also defined as "lamina dura" and it is visible in radiography as a thin line with high radiopacity; Sharpey fibers, coming from cementum in the direction of the alveolar bone, take insertion through lamina dura. *Cortical Bone* is composed by lamellar bone and it defines buccal and lingual/palatal borders of alveolar process. Cortical width variates: it is higher in palatal side of upper jaw or in vestibular sides of anterior mandibula, while it is thinner in the vestibular side of maxilla or in the lingual plates of lower molars. Cortical and alveolar bone are in continuity in the most coronal side of alveolar process, in interdental spaces. Woven bone is the portion of bone included between cortical and alveolar bone, it is also called trabecular bone because of its three-dimensional disposition of trabeculae.

Bone tissue is a connective specialized tissues characterized by the presence of mineralized matrix which provides protection from external injuries and collaborates in systemic homeostasis as mineral reserve. It is characterized by an extracellular matrix composed of proteins, specialized cells and amorphous substance. Extracellular matrix represents the main fraction of bone tissue, while water is the remaining 10%. Bone matrix is composed by organic components (35%) and inorganic components (60%). The organic part is mainly represented by type I collagen fibers (90%) and others non collagenic proteins (osteopontin, osteocalcin, etc...), plus growth factors, the inorganic parts is composed of calcium phosphate in different forms (hydroxyapatite, calcium carbonate and others ions).   The remaining part of bone tissue is made of cell, in particular osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts. Osteoprogenitor cells are unindifferentiated mesenchymal cells able to differentiate in bone line cells under the stimulus of osteoinductive growth factors. They are mainly located in periosteum and endosteum and they are activated during bone remodeling process. Osteoblasts are mononuclear cells able to produce extracellular bone matrix, also defined

as osteoid when it is not completely mature and mineralized, and they work, during bone deposition, in sort of cells clusters. They derive from mesenchymal stem cells stimulated by growth factors and they may be in activate or inactive form, they are structural part of the bone. Osteocytes are mature osteoblast cells which remain embedded in bone matrix as inactive cells, they are in contact with other bone cells trough small canaliculus containing cytoplasmic extensions. Bone canals are essential for bone dynamic life, oxygen and various anabolic or catabolic product flows through the canaliculi. Osteocytes are not yet able to produce bone matrix, but they regulate calcium homeostasis and play their role as bone mechanical receptors. Osteoclasts are multinucleated giant cells involved in bone resorption, they derive from hematopoietic stem cells under osteoclast inducing cytokines stimulus. Osteoclasts are organized in pits called Howship's Lacunae and contain vesicles and vacuoles with proteolytic enzymes able to degrade bone. From a histologic point of view alveolar bone can be divided in compact and spongious bone. Osteon or Havers system is the functional unit of cortical bone; it is based on a central canal (Havers canal) containing nerves, blood vessels, surrounded by concentric bone lamellae. The newly formed lamellae are situated in the central part of the Haversian system, while older lamellae are founded in the external portion of the cylinder. Free spaces between different osteons are filled with interstitial lamellae and perforating holes (Volkmann's canals) that are able to interconnect osteons. Osteocytes are limited in bone lacunae situated in inter-lamellar spaces. Osteons spacial orientation is guided by functional and mechanical bone loading.

## 1.2    Bone Physiology

Bone is a dynamic system and it varies its anatomical shape in response to physiological, pathological and mechanical influence. Bone remodeling is a physiological process that allows to continuously renew tissue and to maintain systemic mineral homeostasis. During remodeling process old bone is reabsorbed, replaced with bone matrix that will go through mineralization process and will consequently form new mineralized bone. Different species of cells are involved in bone remodeling, in a complex balance between neo-apposition work realized by osteoblasts and osteoclasts resorption. Cooperation between osteoblasts and osteoclasts can be seen in bone multicellular units which are composed by a line of osteoclasts in the resorption portion and a line of osteoblasts on the bone matrix formation front. The remodeling process consists of different phases: resorption, reversal and formation. Resorption is acted by osteoclasts, multinuclear cells promoting bone demineralization and resorption. The reversal phase is the time in bone remodeling when mononuclear cells migrate on bone surface and set the right environment for new bone creation by osteoclast. Mononuclear cells produce signaling proteins that are able to stimulate osteoblast migration and differentiation. In the formation phase the osteoblasts produce new bone matrix until new bone is formed in order to substitute the quote lost in resorption phase.  Resorption stage takes usually 2 weeks, while reversal phase is 4 weeks and bone formation includes the further 4-6 months. This timing is important in our research protocol, we decided to verify bone remodeling at 6 months from the extraction phase that coincides with the starting point of resorption. At 6 months we are expecting to analyze the patients' bone in bone formation phase (Hadjidakis et al. 2006, Clarke et al. 2008, Dunstan et al. 2007).

### 1.3 Morphogenetic proteins and cytokines.

Several signaling molecules are involved in bone remodeling process, they are able to up-regulate or down-regulate bone process, balancing resorption effects and new bone formation. These molecules are of fundamental importance in our research, because they can lead us to describe how bone substitutes can influence bone physiology. Both systemic signaling and local factors are able to influence bone dynamics, but, in order to better understand the series of the events involving bone neoformation in post extraction alveolar socket, it is more useful to describe in detail local factors as morphogenetic proteins and cytokines. Furthermore local factors are mainly responsible for bone remodeling after small local trauma and insertion of bone substitute like what we are trying to describe in the explained protocol. Both morphogenetic proteins and cytokines are molecules able to regulate activation, migration and proliferation of bone cells. They function as signaling molecules and activate target cells when bind to specific membrane receptors. After reaching chemical contact with receptors, these factors lead to a series of intracellular signaling processes ending in the activation of specific genes, that can modify cells phenotype and, consequently, activity. The signaling molecules are usually classified through their origin or in reference with target cells (Hollinger et al. 2011).

*Proteins*:

- ***TGF-β***: transforming growth factors β are a big family of proteins that contribute to cells differentiation and proliferation. They are secreted by platelets, osteoblasts and chondroblasts. At the beginning they are inactive but, after proteolytic scission they are divided in latent parts and active components: the active growth factor. TGF-β binds two specific surface cell receptors and promotes intracellular signaling of SMAD proteins and, furthermore, TGF-β promotes Runx2 expression, a key factor for osteoblasts differentiation. All these factors are able to promote new bone formation (Hughes et al. 2006).

- ***BMP***: bone morphogenetic proteins are members of the family of TGF-β. More than forty BMP are produced by our body, but those which are mainly involved in bone remodeling are BMP-2, BMP-4, BMP-5, BMP-6 and BMP-7. They act by binding bone morphogenetic protein receptor type 1 and 2, that are expressed on cell surface, and, after binding, they stimulate intracellular signaling by SMAD receptor (in particular SMAD

1, 4, 5, 8), leading to intranuclear genic expression that ends into osteocalcine and alkaline phosphatases production. It can be affirmed that BMP stimulates mesenchimal bone progenitor cells differentiation through osteoblastic phenotypes, inducing new bone formation.

- *IGF*: insulin like growth factor are polypeptides with amino acid section similar to insulin and are available in two forms: IGF-1 and IGF-2 binding to six different ligand proteins. IGF-1 is strongly connected to bone metabolism process, it is produced by the liver and binds to IGFR1 receptor; when they are bound they promote intracellular signaling inciting proliferation and differentiation of pre-osteoblastic cells and increase the production of Runx2, type I collagen and ALP (Hollinger et al. 2011).

- *PDGF*: platelet derived growth factors is a family of four proteins (PDGF-A, B, C, D) and they act their roles after binding to two specific cell receptors: PDGFRα and PDGFRβ. The most common formula of PDGF is PDGF-BB, it binds to both surface receptors and after binding, it promotes phospho-kinase and tyrosine kinase activity inside cells. PDGF signal promotes mesenchymal cells migration and proliferation, but it is not clear if it has a direct effect on bone formation as osteoinductive factor (Hughes et al. 2006).

- *VEGF* : vascular endothelial growth factors are parts of a big family including 4 different forms (VEGF-A, B, C, D). They are all produced by osteoblasts, chondroblast and platelets and transmit signals through 3 different membrane receptors. VEGF-A is able to induce new vessels formation in early phase of healing and to promote new vessel organization in later phases. It is involved also in bone remodeling process, it is responsible of oxygen and nutrient factors carrying in the cloth, that are fundamental actors for new bone formation. It does not have any effects on osteoblast promotion and differentiation, but, when osteoblasts are still in site, it helps them by stimulating other osteogenic factors releasing (Hollinger et al. 2011).

- *FGF*:  fibroblasts growth factors are a superfamily of 23 correlated proteins, but the most known proteins are called FGF1 and FGF2, they are involved in osteoblasts promotion and angiogenesis. Moreover, FGF9 collaborates with FGF2 in stimulating mature osteoblasts and for this reason are highly expressed during fractures repairing processes.

*Cytokines* are molecules playing a fundamental role during inflammatory events, able to modulate immunity response, but some cytokine may be also classified as growth factor because they are able to stimulate cells proliferation.

The cytokines that are mainly involved in bone cells differentiation and bone metabolism are:

- **RANK / RANK-l**: Rank is a cytokine that binds its specific membrane receptor called RANK-l (RANK-ligand). When binding is completed, it starts a series of intracellular mechanisms, including MPAK and NF-κβ activation, inducing osteoclasts maturation and proliferation. It can be assumed that RANK-L activation by RANK promotes bone remodeling activity.

- **OPG**: osteoprotogerin is produced and secreted by osteoblast, it is able to link to RANK-l, thus blocking the possibility of a RANK/RANK-l interaction and consequently downregulating bone resorption. When OPG binds RANK-l, NF-κβ is inactivated and it stops the promotion of inflammation. OPG can be considered as a protective factor against osteoclast promotion.

- **TNF-α**: tumor necrosis factor alpha is produced by macrophages, mast cells and t lymphocytes, it promotes macrophages activation through a RANK/RANK-l dependent pathway (Dunstan et al. 2007). It also inhibits osteoblasts proliferation by a decrease in the production of extracellular matrix due to a minor expression of osteocalcin and ALP. It also stimulates IL-6 production.

- **IL-6/ IL-1**: Interleukin 1 is one of the most powerful agent that are able to promote bone resorption, it is secreted by macrophages, endothelial cells, B lymphocytes and fibroblasts in two forms (alpha and beta). It is an inflammatory agent always present during healing phases, it brings to bone resorption and bone matrix degradation (McCauley & Nohutcu 2002). Similarly IL-6 is a pro inflammatory cytokine and promotes bone resorption. Both interleukins work by activating RANK/RANK-L pathways, but it is important to underline how different interleukins (i.e. IL-4, IL-10, IL-13) may have different roles and in some cases are able to inhibit bone remodeling processes.

## 2. Alveolar Socket Preservation

### 2.1 Alveolar process after tooth extraction

The alveolar process is a dynamic bone structure that persists in its original morphology until the tooth remains in place; after the tooth loss it goes into remodeling process ending in a complete new management of bone macro morphology. The shape of alveolar process is determined by functional stimulus transmitted by tooth and periodontal apparatus to the bone; when a tooth is extracted or lost for pathological reasons, the bone remains without biological stimuli and the periodontal lost ligament leads to atrophic situation consisting in dimensional reduction of bone crest, both in vertical and horizontal dimension (Atwood 1957, Hedegård 1962). The situation of bone remodeling after tooth extraction may be further worsen when pre-existing bone pathologies are present in site (periodontal o endodontic diseases) or when an incorrect or excessive traumatic bone extraction technique has been used (Van der Weijden et al. 2009). Therefore the correct management of alveolar socket healing is fundamental for a correct implant supported rehabilitation technique that should provide excellent results both from an aesthetic and a functional point of view. The preservation of an important quote of alveolar bone will allow to place the implant in the correct site and with a correct diameter and depth (Vignoletti et al. 2011). Different techniques and materials have been proposed to preserve alveolar bone dimension and the majority of them will be explained in detail later. It is important to remember that in this case the alveolar socket preservation technique represents an in vivo validated model to evaluate bone remodeling pattern and it is not the scope of the authors to evaluate which technique or biomaterial is more useful to obtain bone preservation.

### 2.2 Physiological Healing

Alveolar bone healing after tooth extraction is a fundamental matter in implant dentistry and for this reason, it has been widely studied in animal models and in humans. In animals, in the first period after tooth extraction, mainly in the first week, the remaining periodontal ligament and bundle bone are going to be completely lost. As previously sad, when the tooth is extracted the loss of the periodontal apparatus can be observed and,

consequently, the specific components of periodontal system disappear; cementum is lost with the extracted tooth and bundle bone, remaining periodontal ligament and specific gingiva will be remodeled and substitute with other tissues (Araujo & Lindhe, 2005). Blood cloth fill the empty spaces inside the bone defected left by the extracted tooth and it is sooner replaced by a highly vascularized granulation tissue. The granulation tissue is rich of new vessels, cells of inflammation and extracellular matrix, and the adjacent tissues show high presence of activated osteoclasts and multi nucleated cells (Amler 1969). Starting from the end of the first week of healing, filling tissue is progressively substituted by non mineralized bone matrix. Bone matrix apposition starts from apical and lateral side of the defect and, after 3-4 weeks the new bone matrix occupies the 2/3 of alveolar defects. More in details it has been observed the presence of an osteoblasts line of cells on defects side that tends to include new bone matrix in the central part of bone defect, but, in the outside areas of alveolar bone, osteoclasts cells can be observed. These concomitant factors may explain why, while in the inside part of the alveolus is usually observed new bone formation, it is often described the lost of the thin vestibular cortical plate, due to osteoclasts process of remodeling in the external part. After these initial phases non mineralized bone matrix starts to get mineralized and, progressively in an external to internal dynamic, the matrix evolves in new woven bone. This process can last for several weeks, usually from 4 to 8 after tooth extraction. In the majority of the cases after six weeks it was possible to observe new mature bone and new trabeculae.

During this bone remodeling process, it can be observed at the same time, the wound healing process of gingival tissues. From the 4th day to the 3rd week, epithelial cells migrate from the margins of the alveolus in concentric direction, lining over blood cloth and granulation tissue, and evolve in new keratinized gingival tissue.

Considering human models the situation observed is slightly different, it can be said that in human the healing pattern is slower than in other animals as dogs. Alveolar bone remodeling in human is well described by Trombelli et al. (2008). The authors collected human biopsies at different time points to describe human physiological healing after tooth extraction. In the group of biopsies collected in early phase of healing (2 to 4 week after extraction) the 36% of the tissue was composed by granulation tissue, while in the sample taken in later phases was not found any presence of this tissue. The provisional connective tissue found in all time points was composed by a matrix full of mesenchymal

cells, vascular structures and osteoblasts disposed near vessels and it varied in percentage during the timeline, remaining in a range between 50 and 60 %. The bone matrix was visible starting from second week and was characterized by the presence of osteoclasts and osteoblasts, with osteoclasts mainly present inside Howship lacunae. Mature bone, instead, was not found in any sample with the exception of one collected between 12th and 14th week. It was evident from this study that there was a high inter-patient variability of the remodeling pattern and the reparative process was in every case significantly slower than what observed in dogs.

Furthermore, in Trombelli et al. it was not only completed a histomorphometrical analysis, but some immunohistochemical were performed in order to describe more in detail the biological process. Using CD31 the authors described the variation of endothelial cells in wound area and observed how they decreased from 12th to 24th week; this was mainly motivated by the progressive substitution of the highly vascularized granulation tissue with bone provisional matrix. BMP-7 and osteocalcin, on the contrary, showed an increasing expression from initial phases to 6th and 8th weeks and a decreasing expression going to later phases. These results are in concordance with other studies (Spector et al. 2001) describing higher presence of BMP-7 in early phases of healing.


### 2.3 Bone Grafts

To avoid bone tissue contraction after physiological remodeling, different clinical techniques have been studied in order to preserve bone dimension and facilitate new bone formation inside the wound (Fickl et al. 2008).

Various bio-materials are used for alveolar preservation techniques and they can be synthetically divided into three groups: filling materials, growth factors and membranes. The filling materials are placed inside the bone defect and should be able to maintain space, stabilize cloth, induce and promote new bone formation; growth factors should stimulate new bone formation and membranes cover the area of the defect in order to protect inner spaces from epithelial and/or bacterial colonization. The main goals of the preservation technique are to guarantee implant positioning and stabilization, reduce bone loss, reduce the need of a secondary surgery, improve bone dimensions, and provide an efficient aesthetic and functional result.

Bone substitutes act in healing process in different ways, they can promote 3 main processes, but not all the materials are able to perform these 3 ways:

- **_Osteogenesis_**: it means the formation of new bone starting from graft cells. This process is present only when autologous bone is used, because in this type of bone vital osteogenic cells are enclosed;

- **_Osteoinduction_**:  it is the ability of the graft to activate and attract host osteogenic cells, this process is mediated by active molecules that are enclosed in bio-materials;

- **_Osteoconduction_**: it is the property of the material of being a scaffold inside which, host cells like preosteoblast, can proliferate, mature and produce new bone.

All the materials that are use in clinics to prevent bone resorption after tooth extraction are able to maintain the dimensions during time in order to prevent tissue collapse, but the specific characteristics of each materials determine different pattern of healing, ending in different clinical results. In the next paragraph the major products used for this scope will be exposed and explained, focusing in particular on deproteinized bovine bone graft. It was decided to focus on deproteinized bone graft because, despite it is the most used and described biomaterial, articles that describe his role and his effect on bone dynamics in vivo are lacking.

- **_Autologous bone_**: it is extracted directly from the patient and it can be taken both in intraoral site like maxillary tuberosity or in the mandibular and in extra oral site as iliac crest or calvaria. The autologous grafts do not have any problem of biocompatibility or immunologic reaction and they have osteogenic properties. They are able to promote by themselves new bone formation because they contain living osteoblasts and stamina cells, but are also able to function as space maintaining scaffolds. Autologous bone is remodeled in time and completely substitute with newly formed bone. It could be the best graft, but the morbidity linked to extraction sites and the limited dimension available, tend to limit its use.

- **_Allogenic Grafts_**: it means grafts derived by other human which are frozen, sterilized by means of radiation and chemical agents and finally stored in specific banks. These grafts have a very low risk of antigenic effects and are able to promote new bone formation by both osteoinductive and osteoconductive properties (Mellonig et al. 1981). These grafts

are very easy to obtain in great quantity because they are available in biologic bank stores but, due to the process they received to make them lose their immunologic properties, they have lost also a great amount of their osteoinductive ability.

- **Xenogenic grafts**: the xenogenic graft derives from donors of different species. The most common grafts come from bovine, porcine or equine species. During sterilization and demineralization process all organic components of the grafts are lost, and the remaining demineralized portion functions as scaffold which provides an environment where new bone cells coming from the host are able to proliferate. Furthermore these grafts work as a calcium deposit. The main advantages of xenotrapiants are high availability, osteoconductive property and the lowest risks of immunologic reaction and cross infections.

- **Alloplastic grafts**: these grafts have a synthetic origin and they should be, as other grafts, completely biocompatible, resorbable, osteoconductive and not inducing immunologic reactions. Hydroxyapatite is one of the most used allograft and it is an inert material with osteoconductive ability, it has a slow rate of resorption and functions as a calcium reserve in new bone formation phase. Beta tricalcium phosphate ($\beta$ –TCP) is a porous form of calcium phosphate, it is completely resorbed in 6-18 months, but has a poor ability in space maintaining, for this reason it is conventionally used in association with other materials or autogenic grafts. Bio-active glasses, HTR polimer and algipore are other examples of alloplasti grafts.

The use of the previously described biomaterials is still controversial, some studies have been conducted to verify if the presence of bone graft represents a benefit or not, for the healing process. Nahles in 2012 compared post extractive sites treated with biomaterial or leaving blood cloth alone, evaluating the osteogenic potential of the post extractive site at 4-6 months by means of immunohistochemical analyses. From their results appeared that osteoblastic activation was higher at 4 weeks after healing in both groups and they observed an initial new formation of bone in the apical part of the defect and a consequent migration in coronal direction through time until 12 weeks. It wasn't possible to find any correlation between biomaterial and different healing situation by analyzing CBFA1/runx2 proportion and osteocalcin or CD31.

## 2.4 Membranes

Membranes are fundamental for guided bone regeneration because they work as barrier to protect bone defect from epithelial cells colonization. Melcher in 1976 first described the theory of guided bone regeneration by membrane; it explained that, due to the fact that epithelial cells move and grow faster than other species, if a barrier membrane is not applied the periodontal defect it may be colonized by gingival cells without completely periodontal regeneration. This principle works correctly in periodontal regeneration, but in alveolar socket preservation the biological process is different because, independently from the presence of a membrane, alveolar socket is filled by new bone. The concept previously described is based on the stability of the blood cloth. When the blood cloth is stable in alveolar socket, epithelial cells migrate over granulation tissue and form a new epithelial bridge over bone (Wikesjo et al. 1992).

The question in alveolar socket preservation is on the utility of barrier membrane and on the ability of barrier membrane to improve spontaneous healing. Lekovic et al. (1997) evaluated the efficacy of non resorbable barrier membrane (e-PTFE) for maintaining tridimensional alveolar bone dimensions. They compared 10 spontaneously healed sites and 10 healed with the membrane coverage and found a better healing in test sites, with better maintenance of hight and width. This positive result can be motivated by the space maintaing ability of titanium reinforced barrier membrane, as suggested by Polimeni et al. (2005), but this quality leads to other negative effects. The titanium reinforced barrier membranes need to be removed in a second stage and furthermore, if a membrane exposition occurs, it is rapidly colonized by bacteria and might cause an acute infection of the alveolar socket during healing. For this reason Lekovich et al. (1998) evaluated the efficacy of resorbable membrane (lactide polymers) in a similar model, they compared test group with membranes and control without. The results were similar to what previously observed with non resorbable membrane: they obtained better bone filling and better space maintenance in test group.

## 2.5 Growth Factors

To emphasize the efficacy of the previously described biomaterial, different growth factors were adopted as additional substances to biomaterials. The most used factors are platelet derived growth factor (PDGF), fibroblast growth factor (FGF), bone morphogenetic proteins (BMP) and insulin like growth factor (IGF). Howell in 1997 demonstrated the efficacy of rhBMP-7 in a collagen based carrier and found significant improvements in cortical bone height in alveolar socket preservation procedures. Nevins (2005) demonstrated the efficacy of PDGF-BB in bone defect regeneration in animal models. Despite these positive results, the use of growth factors in alveolar socket regeneration is still very limited, but it may have more applications in the next future.

**Research**

## 3. Rationale

Bone remodeling biologic pattern is well defined in several in vivo and in vitro studies as illustrated in the previous pages, but the influence of bone substitutes on individual healing pattern in human is not completely detailed and described. In order to avoid invasive procedures necessary for autologous bone collection, bone substitutes have been widely used and described in periodontal regeneration, socket preservation and bone regeneration technique. Deprotenized bovine bone (Bio-Oss®, Geistlich Pharma AG, Switzerland) (DBB) is a bovine substitute derived from bovine bone deprived of all the organic components and reduced in porous grains of different dimensions (0.25 - 2 mm) (Gross 1997). Preclinical studies widely described the biocompatibility of deprotenized bovine bone and the integration of this biomaterial in the regenerated bone (Isaksson 1992, Berglundh & Lindhe 1997, Araujo et al. 2008, 2009, 2011) and various clinical papers and reviews proved the clinical benefits of this graft in regeneration procedure (Trombelli et al. 2002, Mardas et al. 2010, Urban & Lozada 2010, Stavropoulus & Karring 2010, Silvestri et al. 2010, Nevins et al. 2006, Esposito et al. 2008, Baldini et al. 2011). The initial histomorphometric data on Bio-oss remodeling were described by Becker et al. (1998). The authors reported that, 3 to 7 months later of DBB placement in fresh extraction socket, the DBB particles were found surrounded by connective tissue with marginal presence on new woven bone. Carmagnola et al. (2003) tried to verify these data on 21 post-extractive socket and found a result comparable to Becker et al.; the DBB at the 9 month evaluation was observed to be comprised in a connective tissue with small percentage of new woven bone, the central part of the grafted site was characterized by DBB particles with surrounding connective tissue, but the peripheral part showed new bone formation. The authors observed also that the quality and the quantity of the bone in the DBB group was clinically sufficient for a correct implant placement, the dimension of the extraction socket site was preserved. Rasperini et al. (2010) in the same clinical model reported that at 3 months the DBB was still in a remodeling phase surrounded by connective tissue and at 6 and 9 months the DBB particles still remained in site, but included in mature new formed bone. Sartori et al. (2003) reported the rate of Bio-oss resorption in a clinical case of sinus lift with histomorphometric analysis at 8 months (29.8% of autologous bone and 70.2% Bio-oss), 2 years ( 69.7% autologous) and 10 years (86.7% autologous), underlying the long term scaffold ability of the DBB graft. Mordenfeld et al. (2010) reported similar data on the 11 year histomorphometric analyses

of 11 patients treated with sinus lift procedures using Bio-oss (80% Bio-oss, 20% autologous), it was found 44.7 +/- 16.9% of lamellar bone, 38+/-16.9% of marrow space and 17.3+/-13.2% of residual DBB.

The reason of this low rate of DBB graft resorption observed in vivo was also investigated in vitro, evaluating genic expression of osteoblast induced by the presence of Bio-oss particles in early phase of healing. Kubler (2004) compared 4 different bone substitutes and found that DBB showed a lower proliferation and differentiation potential on osteoblasts. Thurani (2005) reported similar results, the authors observed less alkaline phosphatase activity in cells cultured on DBB substrate and less osteopontin and osteocalcin gene expression than cell growth on other bone substitute. The genic expressions of proteins involved in osteoblast differentiation and proinflammatory cytokines in a culture of osteoblast in contact with DBB were investigated by Amerio et al. (2010). The results showed a significantly lower expression of bone sialoprotein, BMP-2 and BMP-7 in culture with DBB and also the expression of TNF-α and IL-6 was significantly reduced in DBB culture.

These in vitro papers may be models that explain the slow rate of DBB resorption, but no published scientific study in vivo has confirmed these data. Furthermore these in vitro studies evaluated osteoblast cell response in a model that reproduces early healing phase, but any studies have described the effect of DBB on genic expression in late phase of bone remodeling.

## 4. Aim

The aim of the present research is to describe the remodeling pattern of DBB in human socket alveolar preservation in the late phase of healing. The purpose of this investigation is to verify if, five months after extraction, the remaining DBB particles still influence bone remodeling pattern downregulating osteoblast differentiation and cytokine expression, as already described in the previously cited in vitro studies. This hypothesis of low expression of bone remodeling marker due to the presence of remaining DBB particles included in new formed bone, may justify the results clinically and histologically described in literature.

## 5. Materials and Methods

This is a clinical trial designed to describe the DBB remodeling pattern in alveolar socket preservation procedure. After tooth extraction, a biopsy of bone tissue was harvested from the treated site for analysis of histological features and gene expression at baseline. Then the alveolar socket was randomized in sites grafted with Bio-oss and sites left healed spontaneously. Five months after the surgery, at the implant placement appointment, some hard tissue samples were harvested for analysis of histological features and gene expression. These tissue samples were treated at Università degli Studi di Milano, Dept. of Biomedical Science for Health and Dept. of Biomedical, Surgical and Dental Sciences. The expression of ALP, BMP-2, BMP-7, IL-6, TNF-α in hard tissue at baseline and 5 months after surgery was evaluated by immunohistochemical reaction.

## 5.1 Study population

A total of 20 patients satisfying the following criteria has been identified.

Inclusion Criteria:
- necessity of tooth extraction
- age > 18 years old
- signed informed consent
- no relevant systemic disease
- no antibiotics therapy in the previous four months
- non smoking
- FMPS and FMBS < 15 %

Exclusion Criteria:
- pregnancy or lactation woman
- peri-apical endodontics disease
- suppurative periodontal pocket
- history of biphosphonate therapy
- periodontal disease on adjacent tooth
- absence of buccal cortical wall


Informed consent was obtained from all subjects to be entered in the study. In obtaining the informed consent and in the conduct of the study, the principles outlined in the

Declaration of Helsinki on experimentation involving human subjects were adhered to. The study protocol received the approval by Ethic Committee of University of Milan in 20th July 2011. At each visit the clinician evaluated patients for any untoward effects. In case a patient required any treatment during the course of the study, the necessary treatment was provided at the discretion of the clinician and according to the current standard of care.

## 5.2 Randomization

Each patient included in the study needed tooth extraction and implant replacement in one single site. Every patient represents a single unit and was randomly assigned to test group (alveolar socket preservation with DBB) or control group (spontaneous healing). The clinic discovered which technique to use only after tooth extraction, by opening a closed envelope containing indication to test or control. The 20 envelopes were prepared by a blind operator and randomly assigned to the scheduled patients.

## 5.3 Surgical Procedure

The teeth were extracted using a flapless, minimally invasive technique (Fig.1). After tooth extraction bone samples were collected in the lateral side of the alveolus using a trephine (internal diameter 2.0mm, Hu-Friedy, Usa). In presence of an infra-osseous defect the bone sample was taken in the opposite side of the alveolus.
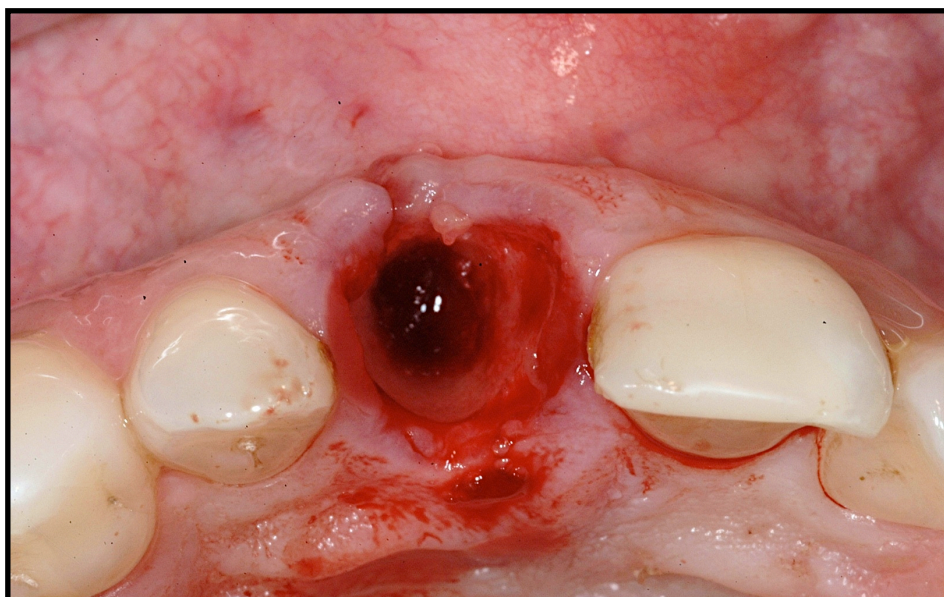
Fig. 1 Infraosseus defect after tooth extraction.

Thus in test group, the hard tissues were curetted, and Bio-oss granules (Bio-Oss® 0.25-1mm, Geistlich Pharma AG, Switzerland) were grafted into the socket up to 2 mm apical to the soft tissue margin (Fig.2). A resorbable membrane (Bio-Gide®, Geistlich Pharma AG, Switzerland) was used to cover the alveolar socket, and a criss cross suture with 5-0 Gore-tex was realized to stabilize the graft (Fig.3).

In control group after tooth extraction the hard tissues were curetted and a cross suture with 5-0 Gore-tex was realized over the extraction site.
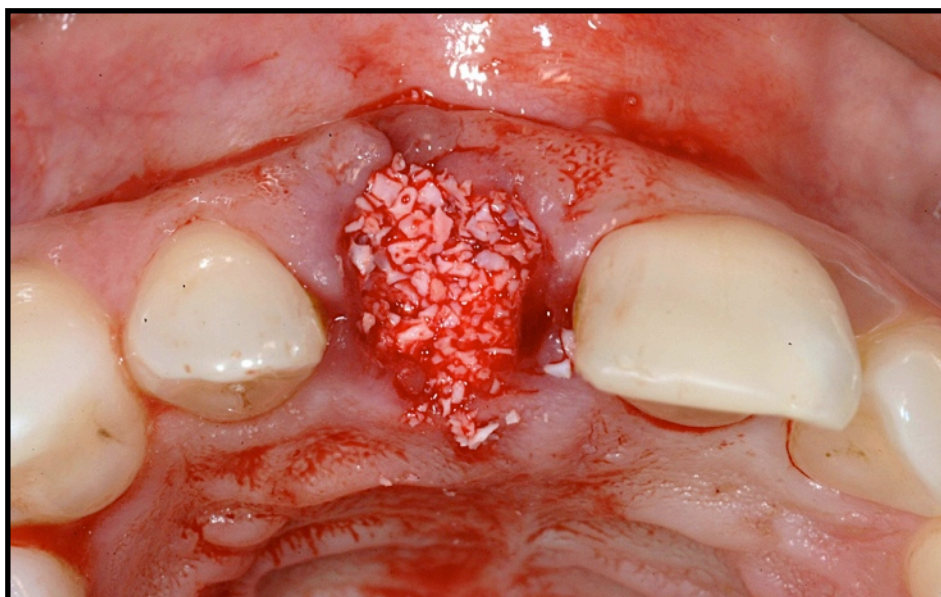


Fig. 2 Alveolar socket grafted with DBB
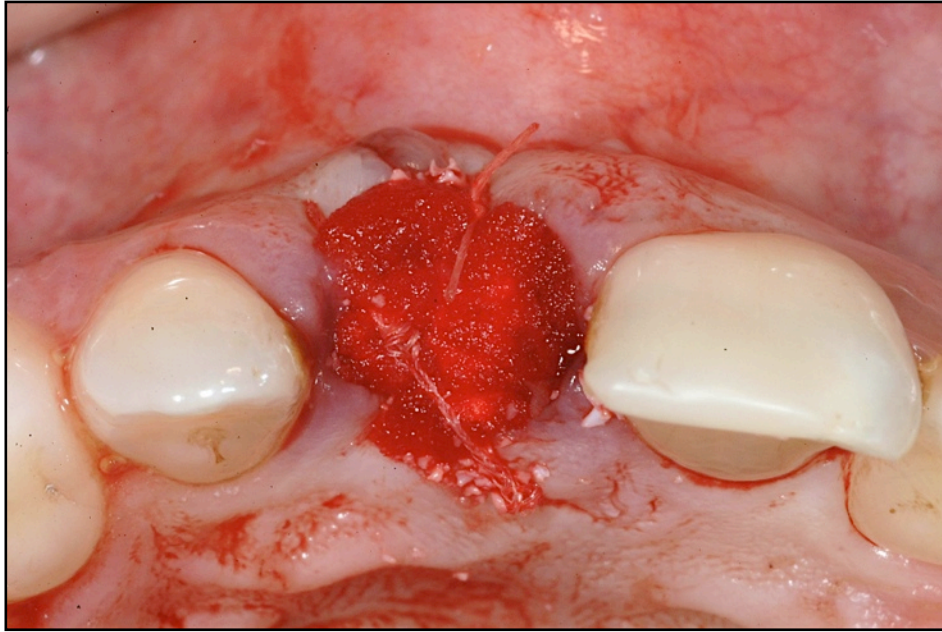
Fig. 3 Alveolar socket closed with sutures.

Post-operative pain and edema were controlled with ibuprofen. Patients received 600 mg at the beginning of the surgical procedure and were instructed to take another tablet 6 hours later. Subsequent doses were taken only if necessary to control pain. Patients with ulcers, gastritis, and other contraindications to NSAIDs received 500 mg acetaminophen. In these cases, the second dose was after 6 hours. All patients were instructed to intermittently apply an ice bag on the operated area (5 min yes, 5 min no, for the first 2 hours). All patients were instructed to discontinue toothbrushing and avoid trauma at the surgical site. A 60 second rinse with 0.12% chlorhexidine digluconate was prescribed 3 times/day for the first 2 weeks.

The sutures were removed after 10 days. Patients were instructed to brush with a post-surgical soft toothbrush for the following weeks, resuming interdental cleaning. Two weeks after surgery, the patients were instructed to resume regular mechanical tooth cleaning of the treated areas using a regular toothbrush with the appropriate technique. Patients were recalled for controls (and prophylaxis as needed) at week 1, 2, 4, 6 and 12 weeks (Fig.4).

Fig.4 Healing.

At the 5 months appointment (T1) a re-entry procedure was performed. After topic anesthesia a full-thickness flap was raised and using a trephine (internal diameter 2.0 mm, Hu-Friedy, Usa), bone specimens were collected before implant placement. After implant placement a single interrupted 5.0 monofilament (Gore-tex) sutures was used for flap adaptation.

At baseline was collected one bone biopsy with trephine int. diam. 2 mm (3 mm depth) (immunohistological analysis) and at 5 months were collected two bone biopsy with trephine int. diam. 2 mm (3 mm depth) immunohistological/histomorphometric analysis)

## 5.4 Histologic Processing

The specimens taken at T0 were immersed in 10% formalin/0.1 mol/L phosphate-buffered saline (pH 7.4) for 24 hours at room temperature and then decalcified for 2 days in ethylenediaminetetraacetic acid decalcification product. After that, decalcification was verified with a radiograph, the bone cores were rinsed with running water for 24 hours, routinely dehydrated in a series of increasing concentrations of ethanol (50%, 70%, 80%, 90%, 96%, 100%); placed in xylol for 12 hours, and then embedded in paraffin. Serial longitudinal sections of about 5 μm were stained with haematoxylin/eosin (Sigma-Aldrich, St Louis, MO). The slices after immuohistochemical staining were observed and

photographed under a Nikon light microscope (Eclipse E600) equipped with a calibrated digital camera (DXM1200, Nikon, Tokyo, Japan).

In each patient two bone biopsy were harvested at T1. One biopsy was decalcified and paraffin-included as described in the previous paragraph, the other biopsy was prepared for light microscopy without prior demineralization in accordance with the method of Donath & Breuner in 1982. In brief, the undecalcified bone samples was fixed in 10% formalin/0.1M phosphate buffer saline solution, dehydrated by increasing ethanol concentrations, infiltrated and embedded in Kulzer Technovit 7200 VLC (Bio-Optica, Milano, Italy). Two longitudinal midsections were obtained per block using a diamond saw (Micromet & LS2, Remet, Remet, Bologna, Italy), grounded and polished (LS2, Remet, Bologna, Italy) to a final thickness of about 50μm. The sections were mounted on plastic slides, stained with toluidine blue/pyronine G (Sigma-Aldrich, St Louis, MO) and observed using a Nikon light microscope (Eclipse E600) equipped with a calibrated digital camera (DXM1200, Nikon, Tokyo, Japan). The undecalcified section was analyzed by histomorphometry. The decalcified part was used for immunohistochemistry.

Histomorphometric measurements of the tissue fractions (marrow spaces, DBB, lamellar bone) in the samples were performed on images at a magnification of 100× using a standard stereologic method. A point-counting grid consisting of 100 test points was placed over each microscopic image section and the tissue underlying each grid intersection were recorded as either new bone, residual hydroxyapatite or bone marrow spaces. The number of hits containing new bone, grafted particles or marrow spaces were separately divided by the total number of possible intersections and thus expressed in percentage values representing the volume density of these 3 components.

On decalcified sections immunohistochemical investigations were performed to identify anabolic (alkaline phosphatase [ALP], Bone Morphogenetic Proteins 2 and 7 [BMP-2], [BMP-7]) and catabolic bone markers ( TNF-α and IL-6).

More in detail, the antigen retrieval was done with Proteinase K solution at 37° in humidified chamber for 20 minutes. Four sections for each sample were incubated at room temperature for 90 minutes with the following antibodies ALP (alkaline phosphatase, 1:100, Santa Cruz Biotech, inc., Santa Cruz, CA, USA), BMP-2 (Bone Morphogenetic Proteins 2, 1:200, Santa Cruz Biotech, inc., Santa Cruz, CA, USA), BMP-7 (Bone Morphogenetic Proteins 7, 1:200, Santa Cruz Biotech, inc., Santa Cruz, CA, USA),

TNF-α (Tumor Necrosis Factors α, 1:100, Santa Cruz Biotech, inc., Santa Cruz, CA, USA) and IL-6 (Interleukin 6, 1:100, Santa Cruz Biotech, inc., Santa Cruz, CA, USA). To have control sections, treatment with primary antibody was omitted in selected sections. Then sections were washed with PBS solution for four times for 5 minutes, immersed in labelling solution (Ultravision Quanto Detection System HRP, Thermo Scientific, Loughborough, Leicestershire, UK) and stained with DAB (diaminobenzidine, Ultravision DAB, Thermo Scientific). All the sections were counterstained with Mayer's haematoxylin.

For each marker 4 sections (2 marked and 2 controls) per specimen were analyzed. All markers were quantified using a specific image analysis software (Photoshop CS5, Adobe System) at a total magnification of 400X and 4X. It was decided a color range that represented marked cells and it was calculated the fraction of marked pixels over total pixels of the entire image.

## 5.5 Statistical Analyses

The aims of the statistical analyses are to compare T0 and T1 in both groups, and to compare test and control normalized difference between T0 and T1. Normalized difference was calculated as (T1-T0)/T0. Statistical analyses were performed with Kyplot software (Kyplot, 2012, KyesnLab INC) : non parametric tests for paired (Wilcoxon signed rank test) or unpaired (Wilcoxon sum rank test) samples were applied with a level of significance of 0.05.

## 6. Results

### 6.1 Histomorphometry

It was analyzed on non decalcified samples the fractions of different tissues. At T1 in test sample it was found  33.4% of DBB, 14.6% of lamellar bone, 25.8% of osteoid and 26.2% of bone marrow. At T1 in control group it was found 30.6% of lamellar bone, 29.3% of osteoid and 40.1% of medullary space. These data are shown in the graphics below (Fig. 5-8).
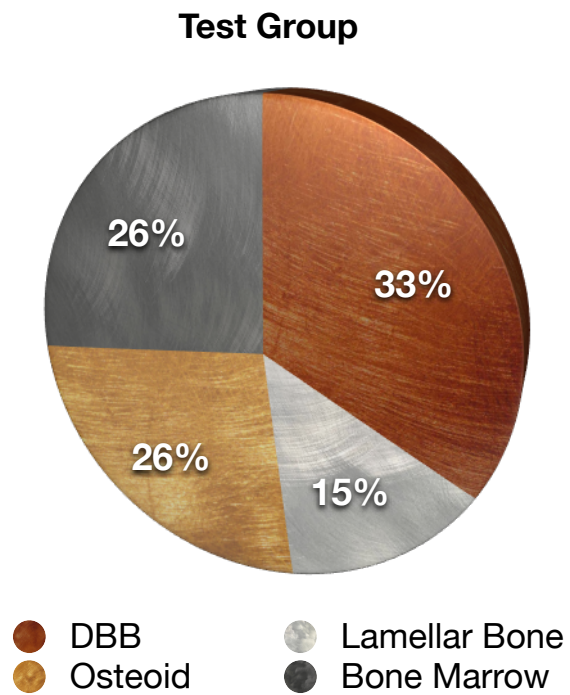
## Test Group



Fig.5 Fractions in Bio-oss group.
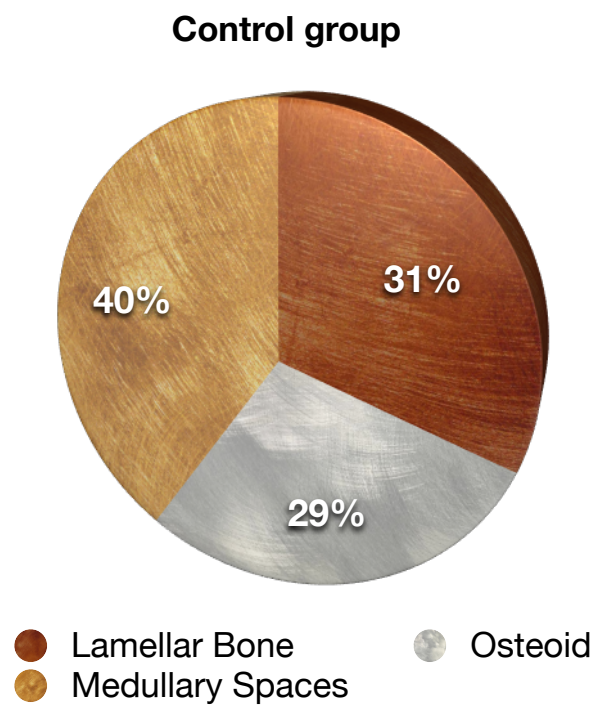
## Control group



Fig.6 Fractions in control group.
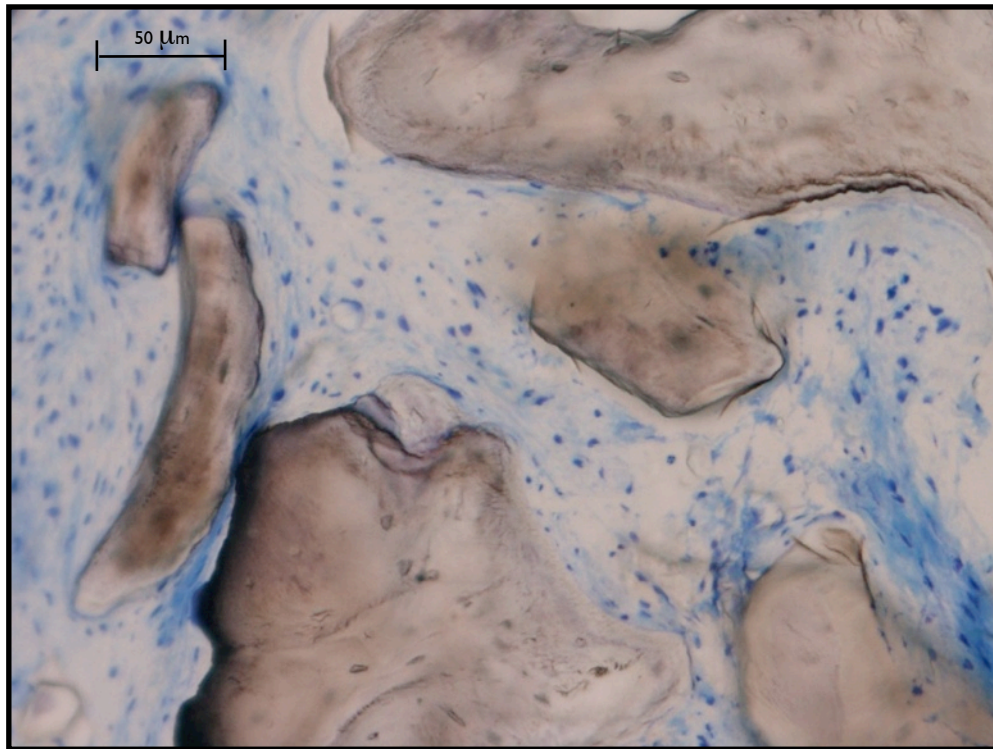
Fig.7 Details at 200X total magnification of DBB particles surrounded by osteoid at T1 in test group.
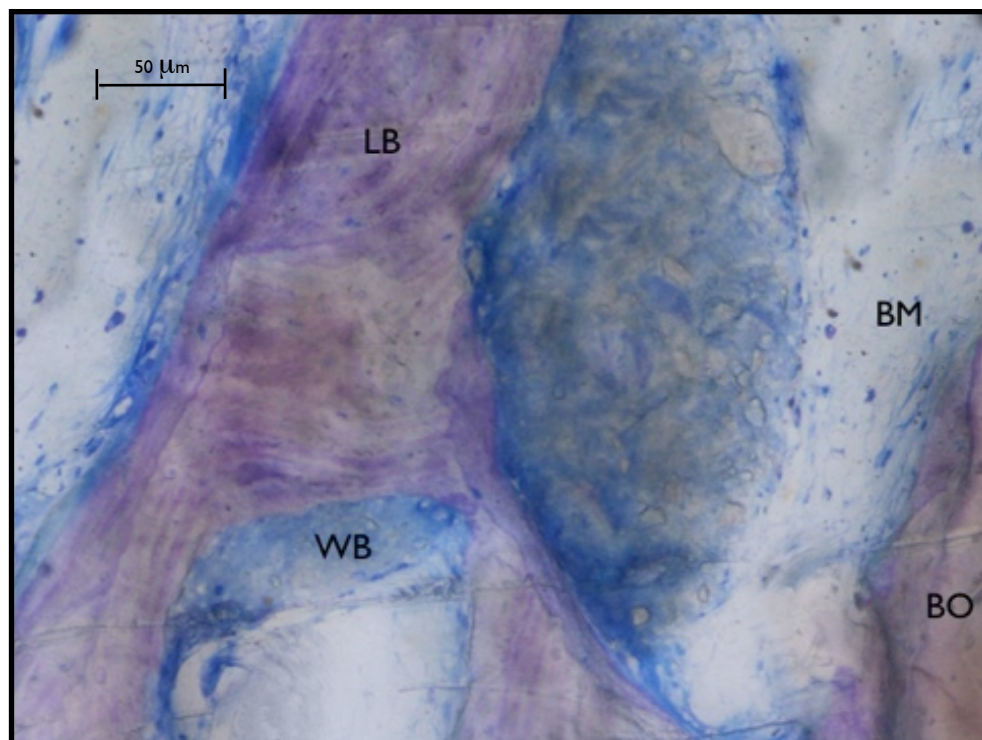


Fig.8 Details at 200 X magnification of T1 in test group. LB: lamellar bone WB: woven bone, BM: bone marrow, BO: DBB.

## 6.2 Hematoxylin & Eosin

The observation at microscope of the non decalcified section stained with hematoxilin-eosin confirmed that all the bone samples at T0 were healthy, without evidence of acute inflammation. After laboratory process the samples morphology appeared to be well preserved, confirming the absence of modification due to eventual procedure errors. It was possible to identify all the morphologic characteristics of the sample: compact bone results well organized in lamellar structure. Osteocytes were stained with violet color and were clearly visible in lacunae inside bone matrix. The medullar spaces appeared rich of collagen fibers and various cells coming from blood vessels. At T1 in the test group biomaterial particles were clearly visible, but without any sign of inflammations. In proximity of DBB particles there was presence of immature bone matrix, which was a clear sign of new bone formation surrounding grafts. The osteoblast that were visible in this area were another sign of bone remodeling process. In the majority of the test sample it was not possible to find high presence of new lamellar bone, on the contrary, in the spontaneously healed control group, it was easier to find new mature bone. Complementary in T1 test group there was more collagen then what observed in T0 or in control cases (Fig.9).
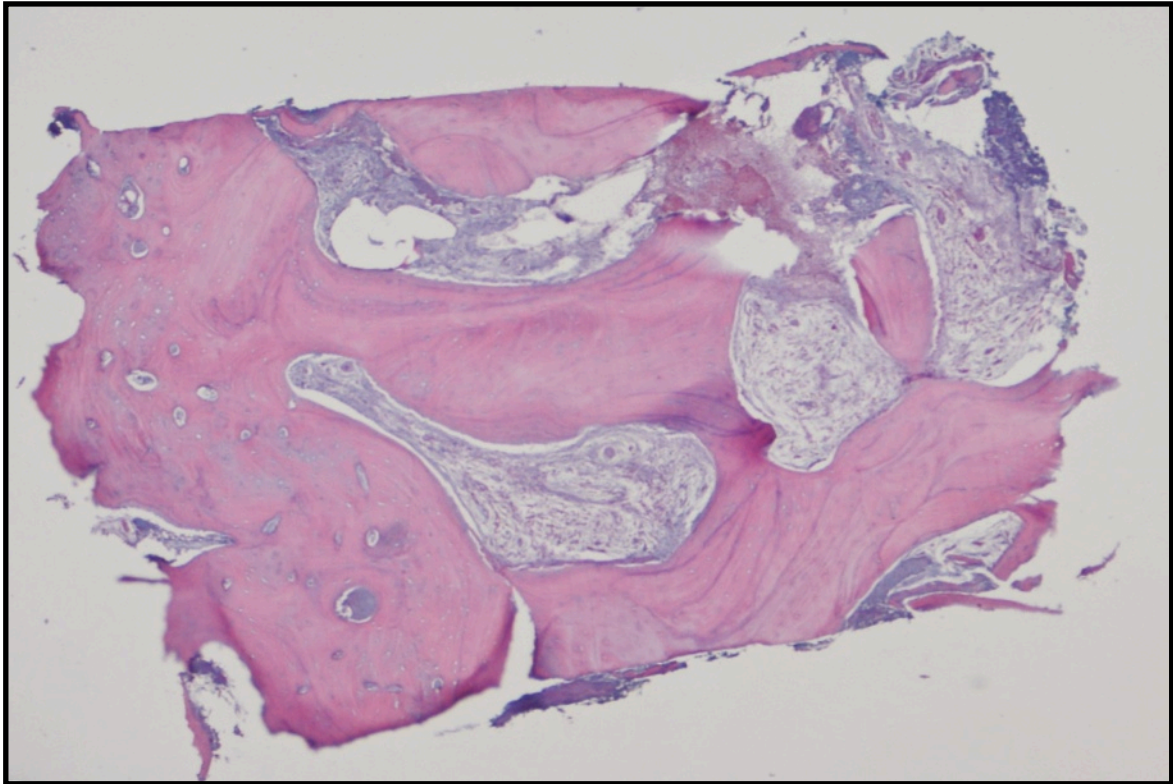
Fig.9 T0 samples with e-e staining.

## 6.3 Immunohistochemistry

For every specimen a semiquantitative analysis was done to evaluate the percentage of marked pixels over total pixels of the images and the mean normalized differences between T0 values and T1 values in both groups were calculated. The results are shown in the table and figures below (Fig.10-12).

| Test | BMP-2 | BMP-7 | ALP | IL-6 | TNF α |
|------|-------|-------|-----|------|-------|
| Mean | + 0.67 | + 0.36 | -0.28 | + 0.81 | + 1.09 |
| S.D. | 0.43 | 0.23 | 0.18 | 0.6 | 0.85 |
| C.F. | 63.74 | 64.22 | 62.65 | 74.39 | 77.82 |
| Control | BMP-2 | BMP-7 | ALP | IL-6 | TNF α |
| Mean | + 1.42 | + 0.1 | -0.23 | + 0.45 | -0.01 |
| S.D. | 0.11 | 0.13 | 0.15 | 0.8 | 1.3 |
| C.F. | 7.62 | 66.62 | 61.97 | 68.43 | 77.82 |

Fig.10 Normalized difference between T0 and T1 value in both groups. S.D. = Standard Deviation C.V. = Coefficient of variation
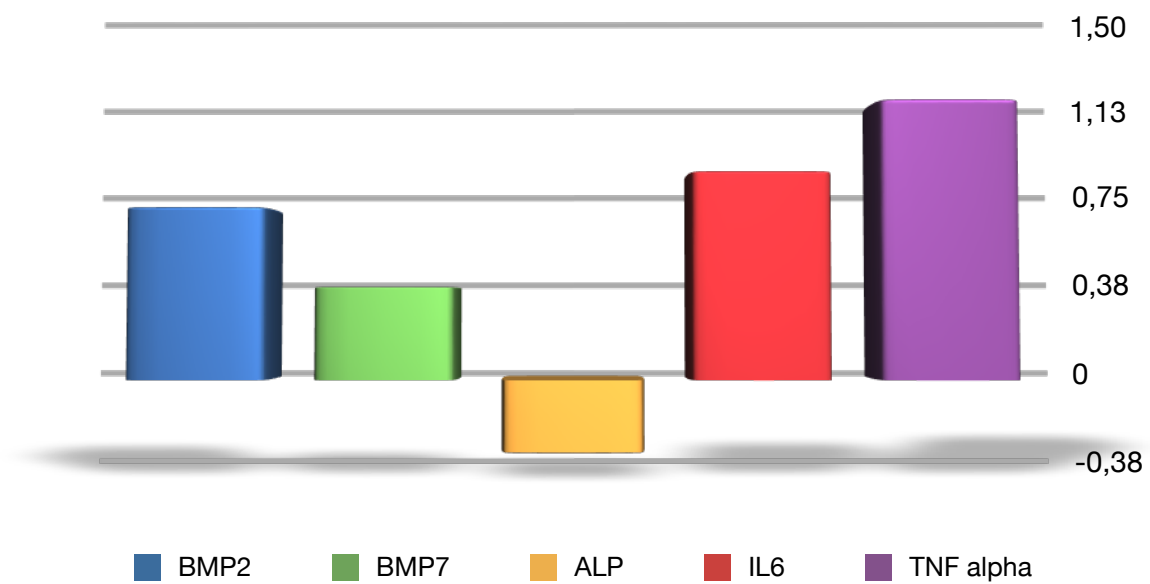
Fig.11 Normalized difference between T0 and T1 in test group
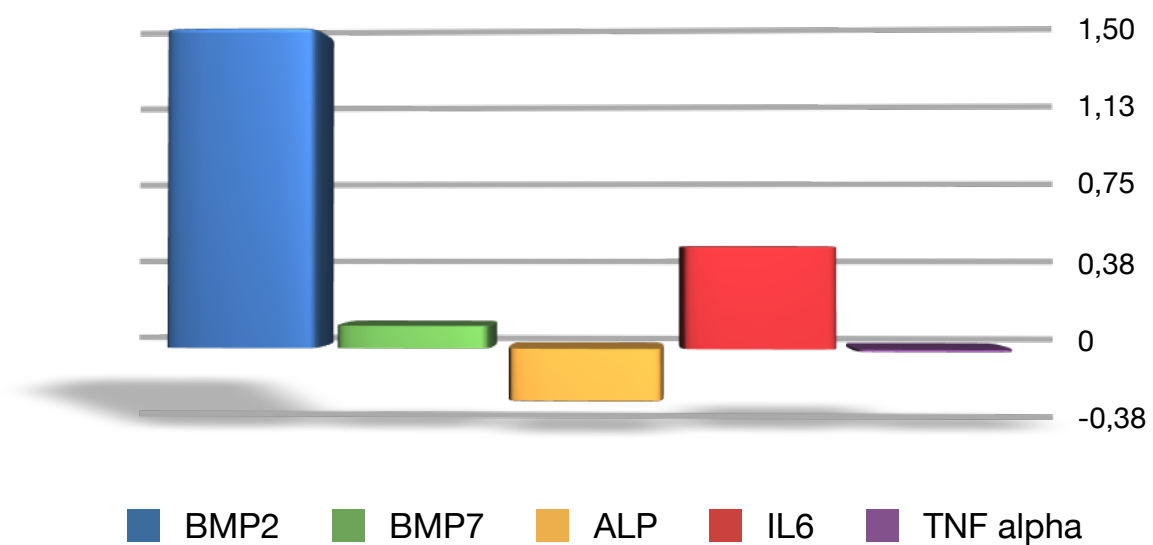


Fig.12 Difference between T0 and T1 in control group

**BMP-2**

BMP-2 marking was clearly visible in both groups at T0 and T1, but in the T0 stages it was prevalent an intracellular marking, while at T1 in both group it was noticed an evident staining also outside cells, near graft in bone matrix and between collagen fibers. Bone mineralized matrix is stained with BMP-2 in all the samples, but it can be seen a denser coloration in both 5 month bone samples.  (Fig.13-17)
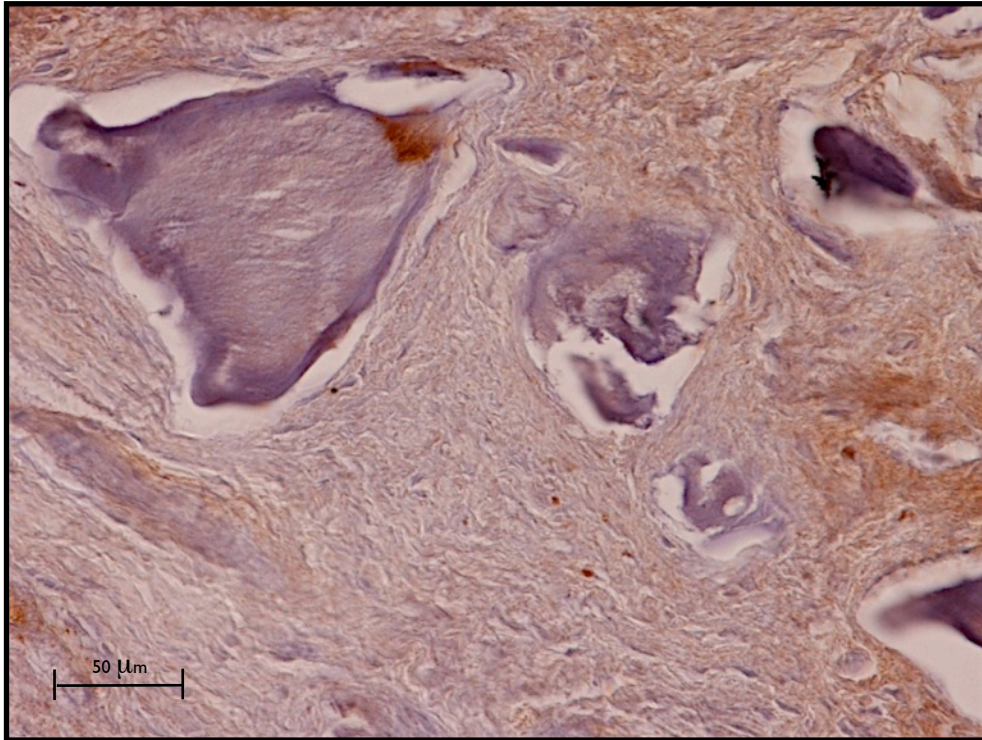


Fig.13 BMP-2 staining in T1 test in proximity of DBB particles. 200X total magnification.
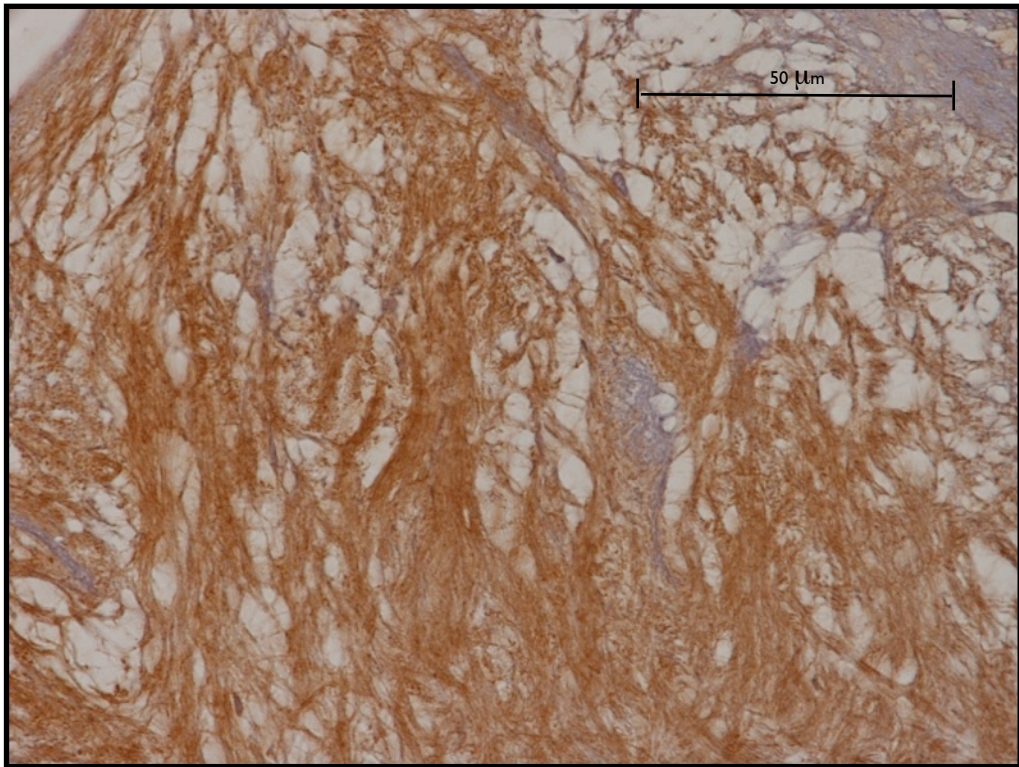
Fig.14 Diffuse BMP-2 staining in collagen matrix at 400X total magnification in T1 Test.
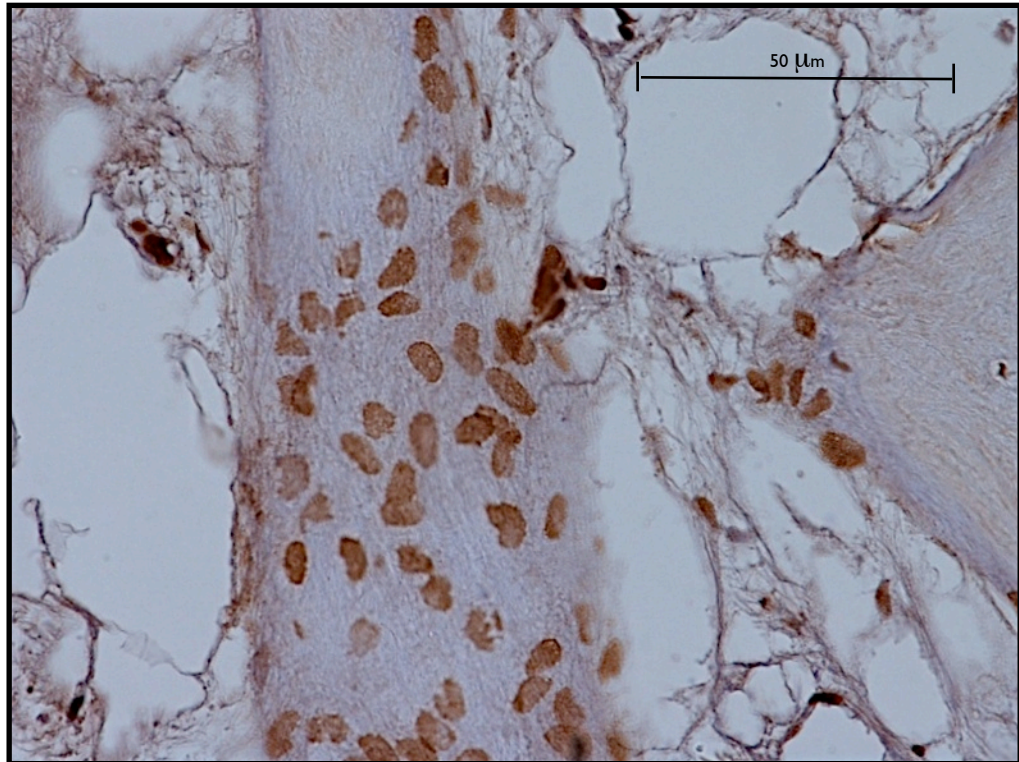


Fig.15 BMP-2 staining at T1 in control group at 400X total magnification.
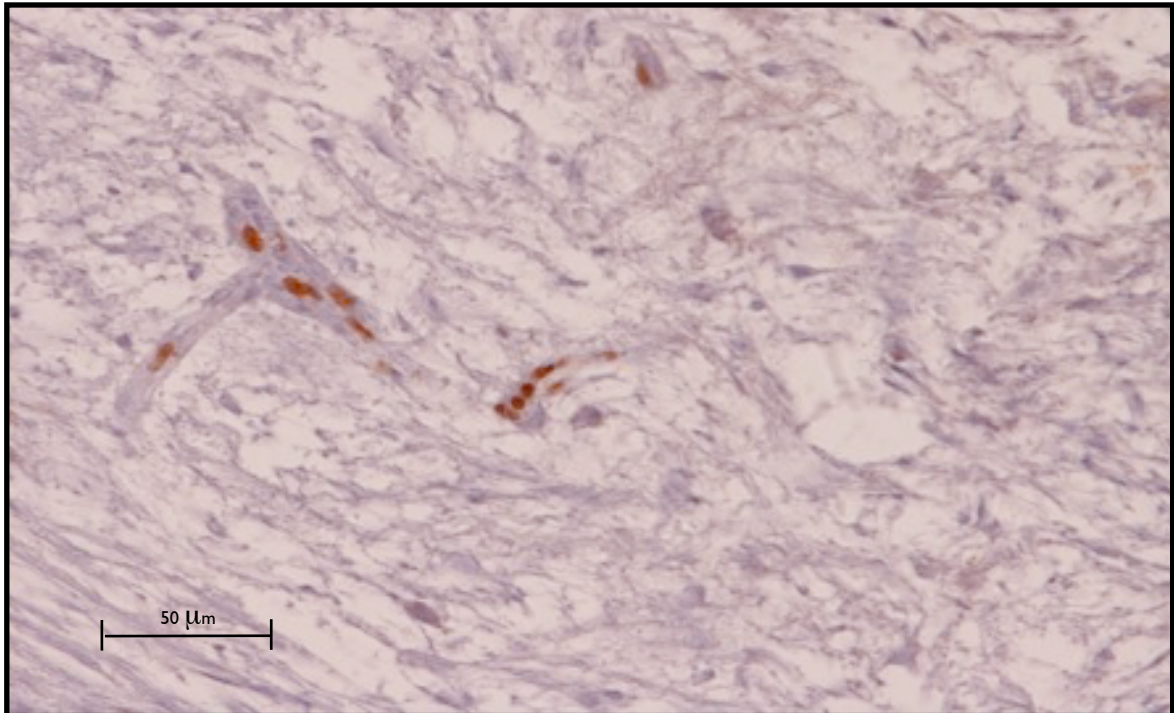
Fig.16 Multinucleated cells marked with BMP-2 at 200X total magnification at T0.
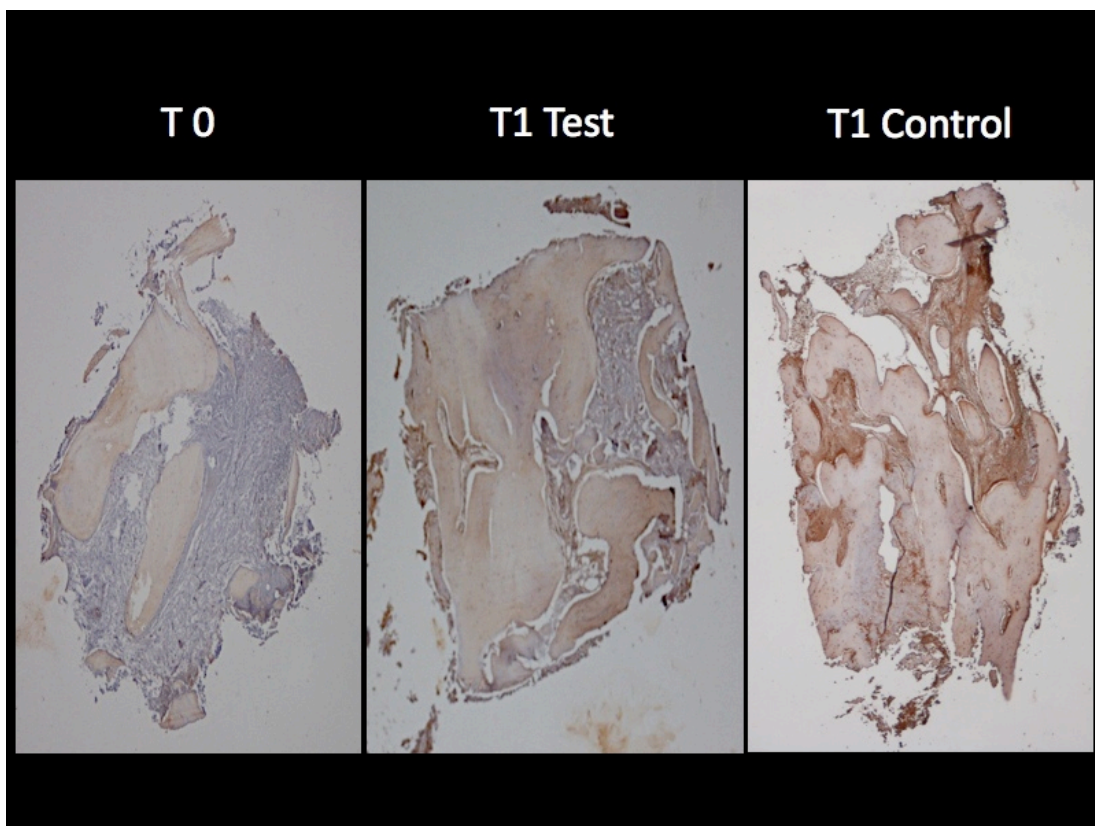


Fig.17 Images of BMP-2 stained samples at T0, T1 in DBB group and T1 in control group. It can be noticed a higher staining in T1 in both groups.

**BMP-7**

BMP-7 marking was denser in bone medullary spaces at T0 and T1 in both groups and was completely absent in lamellar bone, showing a completely different pattern of staining in comparison to what observed with BMP-2. Inside medullary spaces it can be seen both an intracellular and extracellular staining, in particular there is a high presence of positive marking on collagen fibers at T0 and T1 time points. Despite the absent staining in lamellar bone, osteocytes cells, trapped inside mineralized bone, are clearly marked. (Fig. 18-22)
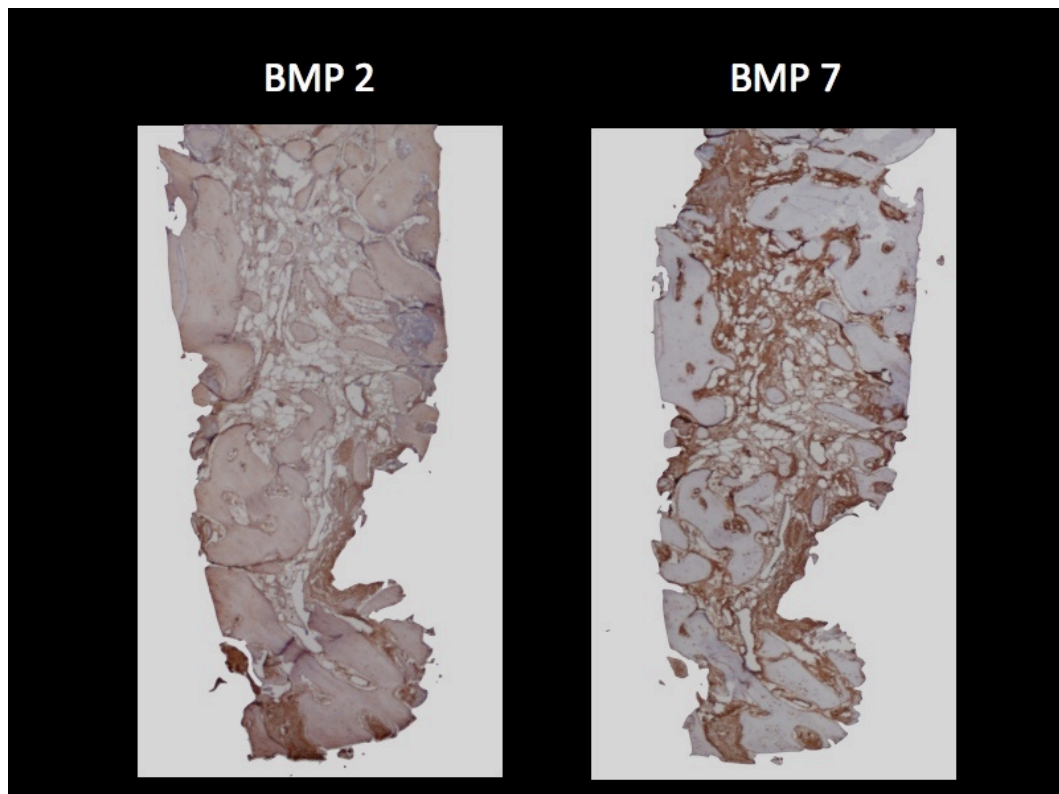


Fig.18 Comparison of BMP-2 and BMP-7 marking in TO samples (first image) and T1 samples (second image)
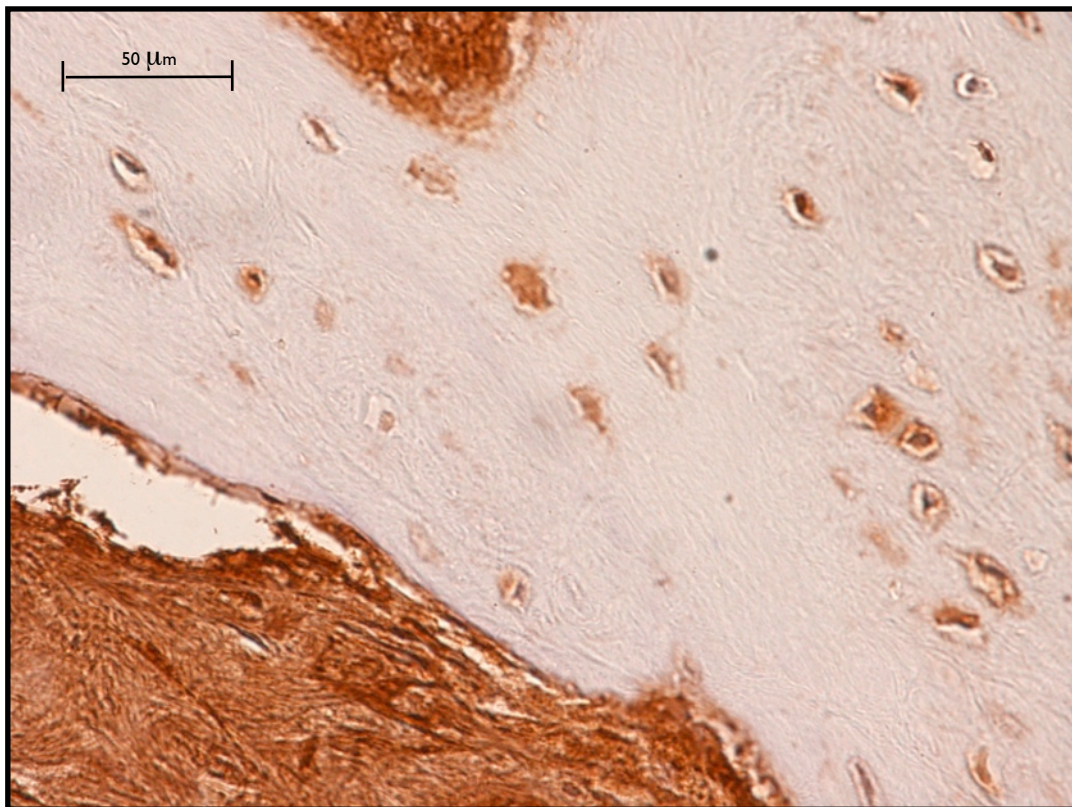
Fig.19 BMP-7 staining at T0, 200X total magnification.
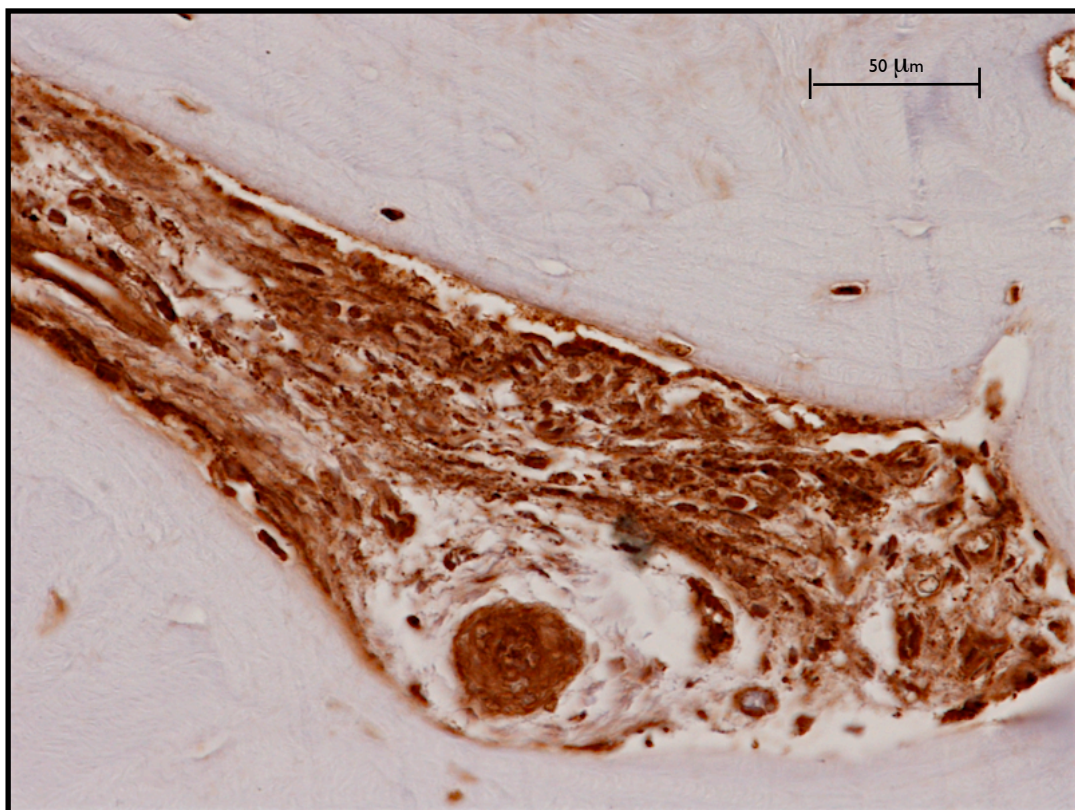


Fig.20 BMP-7 staining at T1 in test group, 200X total magnification
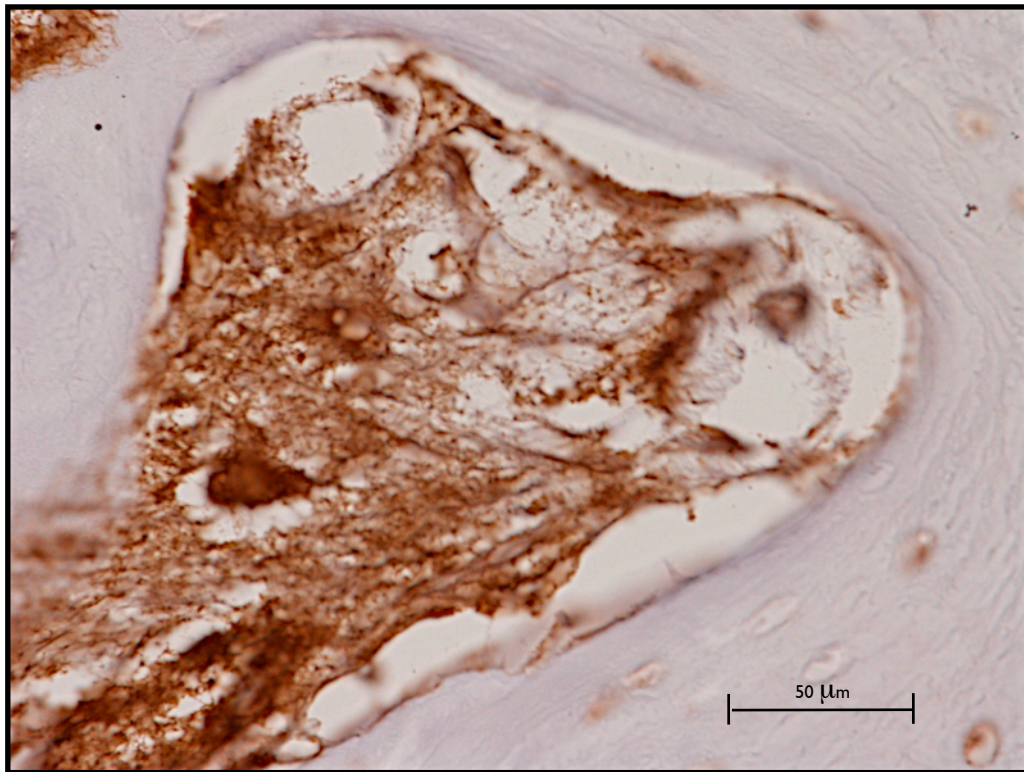
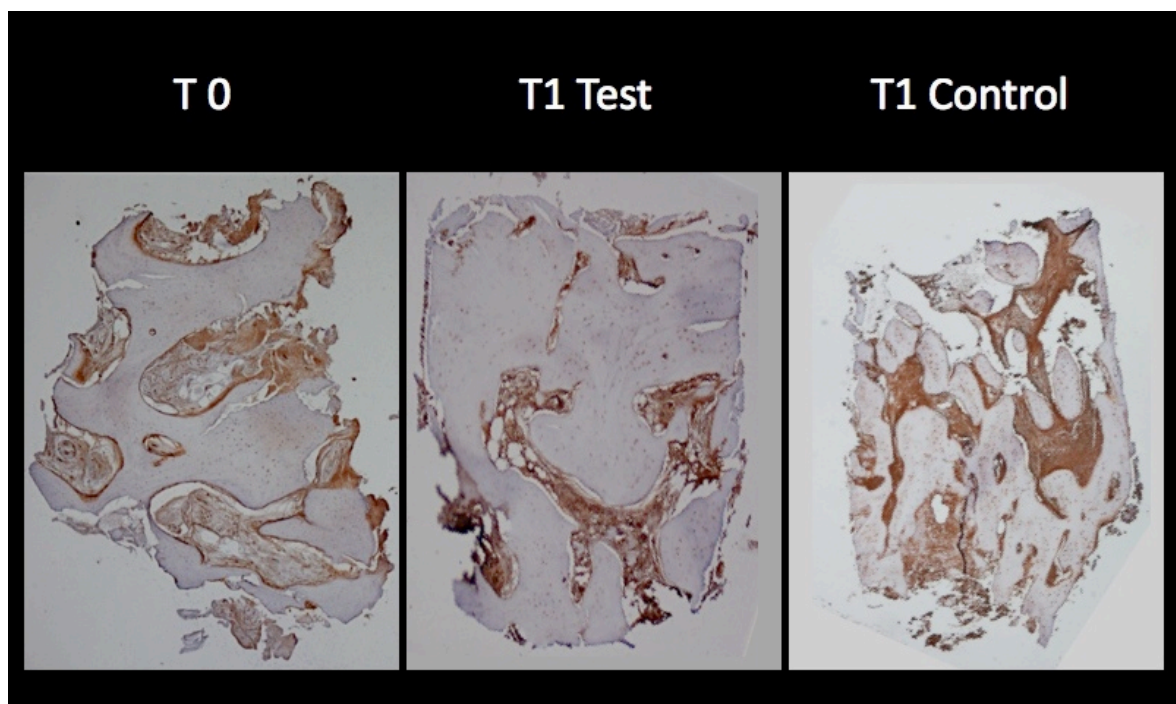Fig.21 BMP-7 staining at T1 in control group at 200X.



Fig.22 BMP-7 marking at T0, T1 test sample and T1 control sample.

**ALP**

ALP is situated, in all the analyzed samples, in the periphery of mineralized lamellar bone, it shows clearly the line of new bone formation. It is absent near the graft particles, confirming the new bone formation in direct contact with DBB (Fig. 23-26).
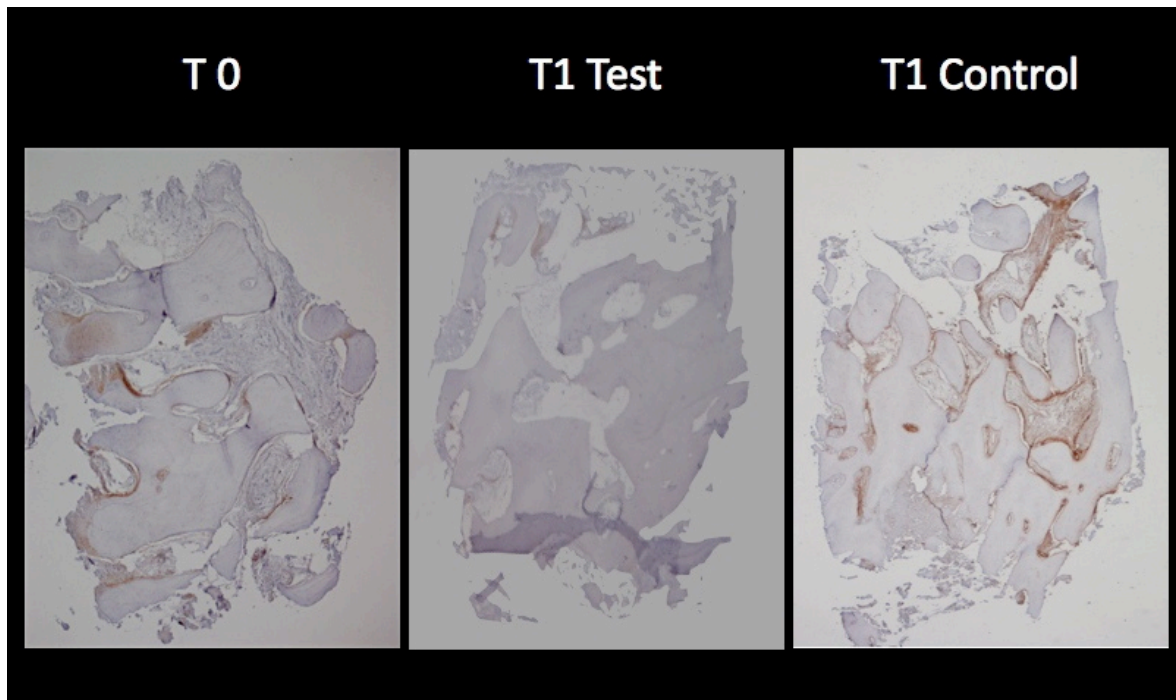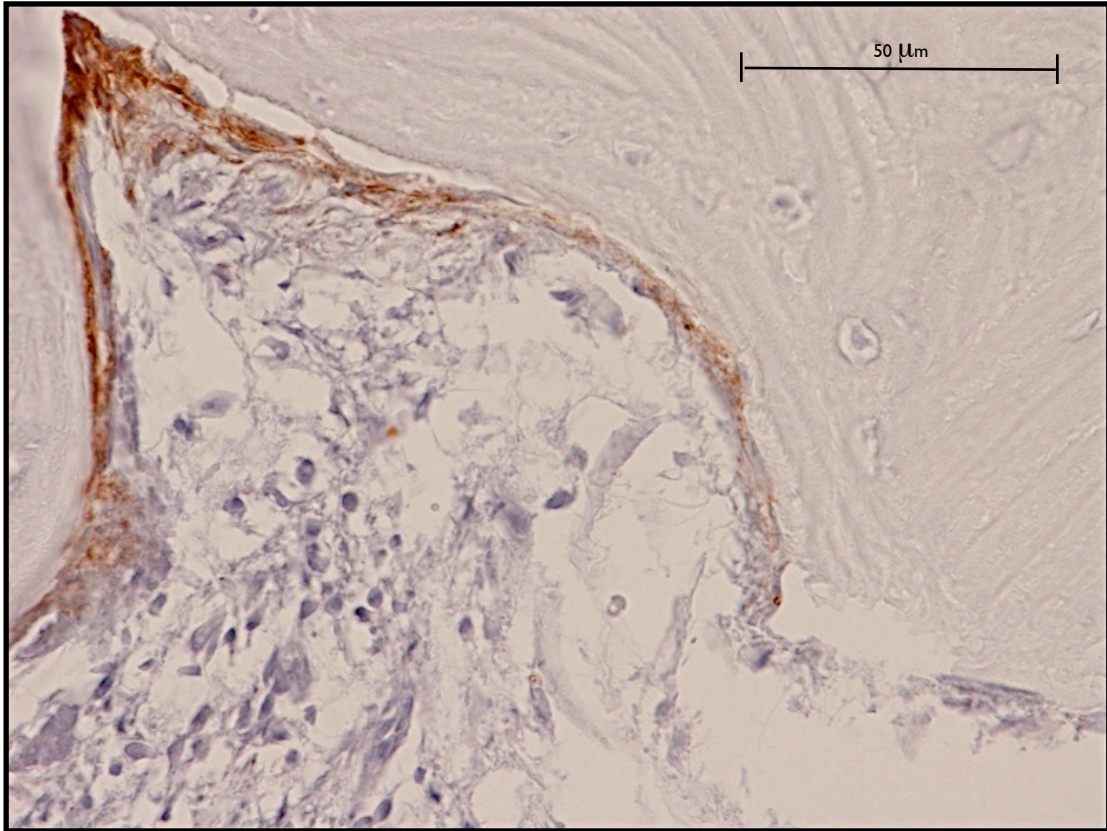


Fig.23 ALP in samples at T0, T1 test and T1 control

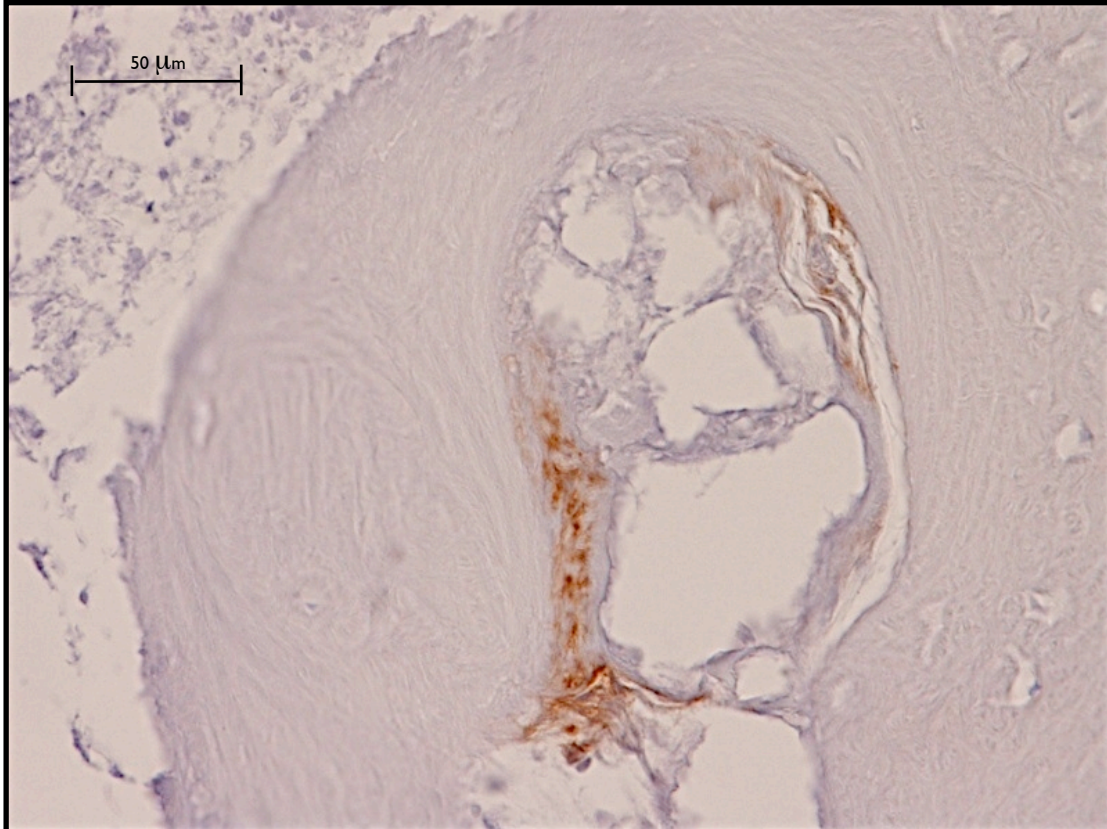Fig.24 ALP marking at T0 at 400x magnification.



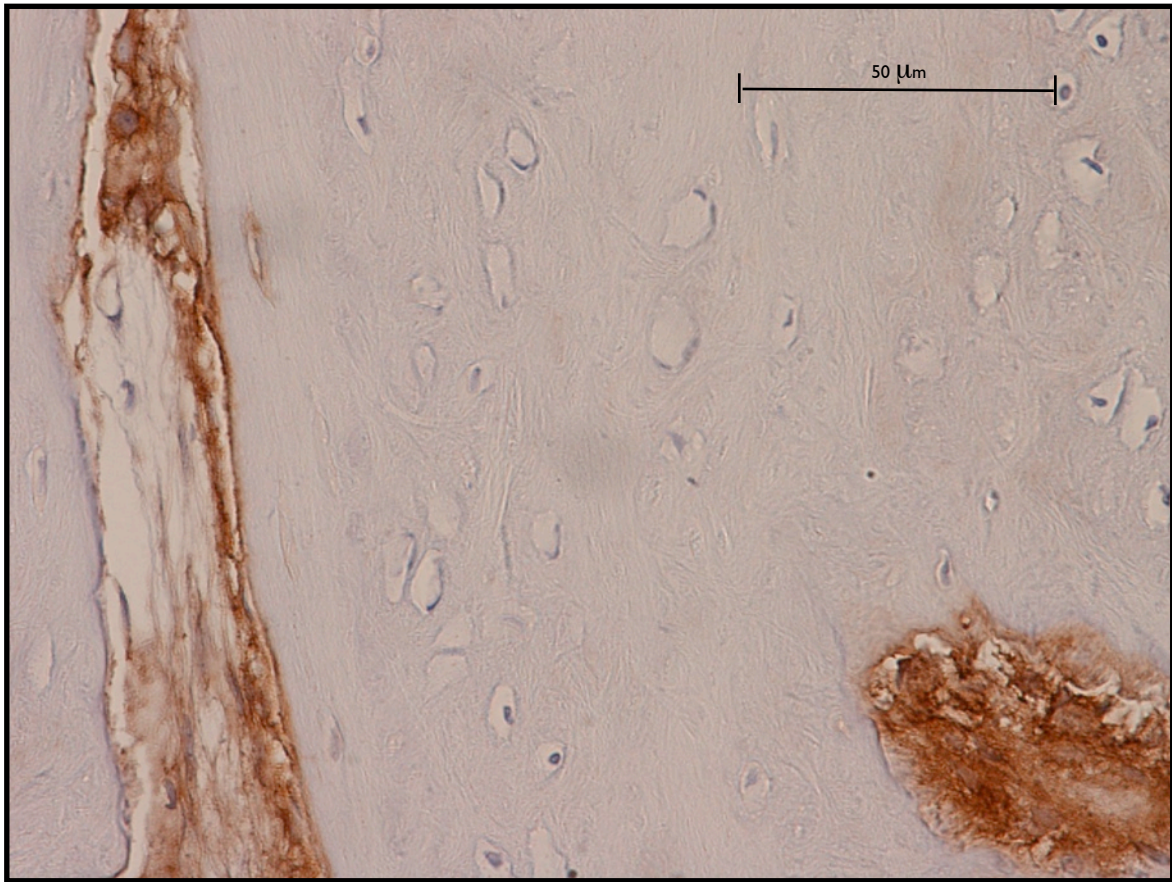Fig.25 ALP marking at T1 at 200X magnification in test samples.

Fig.26 ALP marking at T1 at 400X magnification in control sample.

**IL-6**

Interleukin 6 is mainly distributed in extracellular sides in medullary spaces, and appears to be not organized and diffused in spot between collagen fibers. It is mainly produced by osteoclasts and osteoblasts and should stimulate the bone resorption. For this reason it is visible near the graft particles, in osteoid matrix (Fig. 27-30).
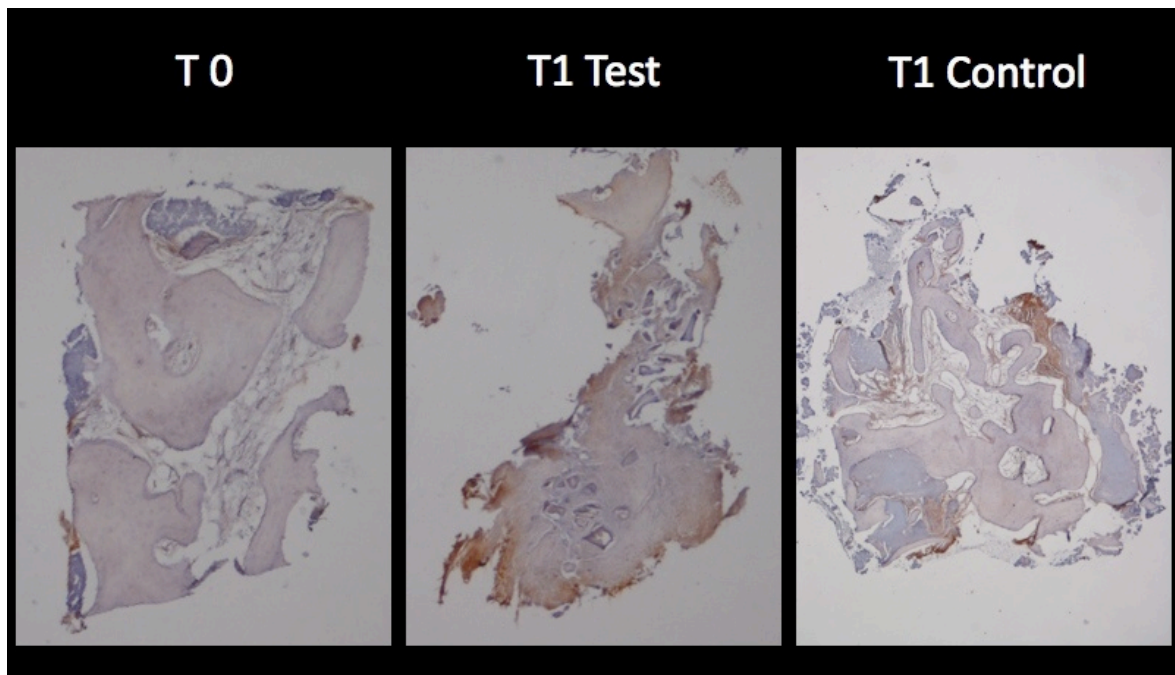
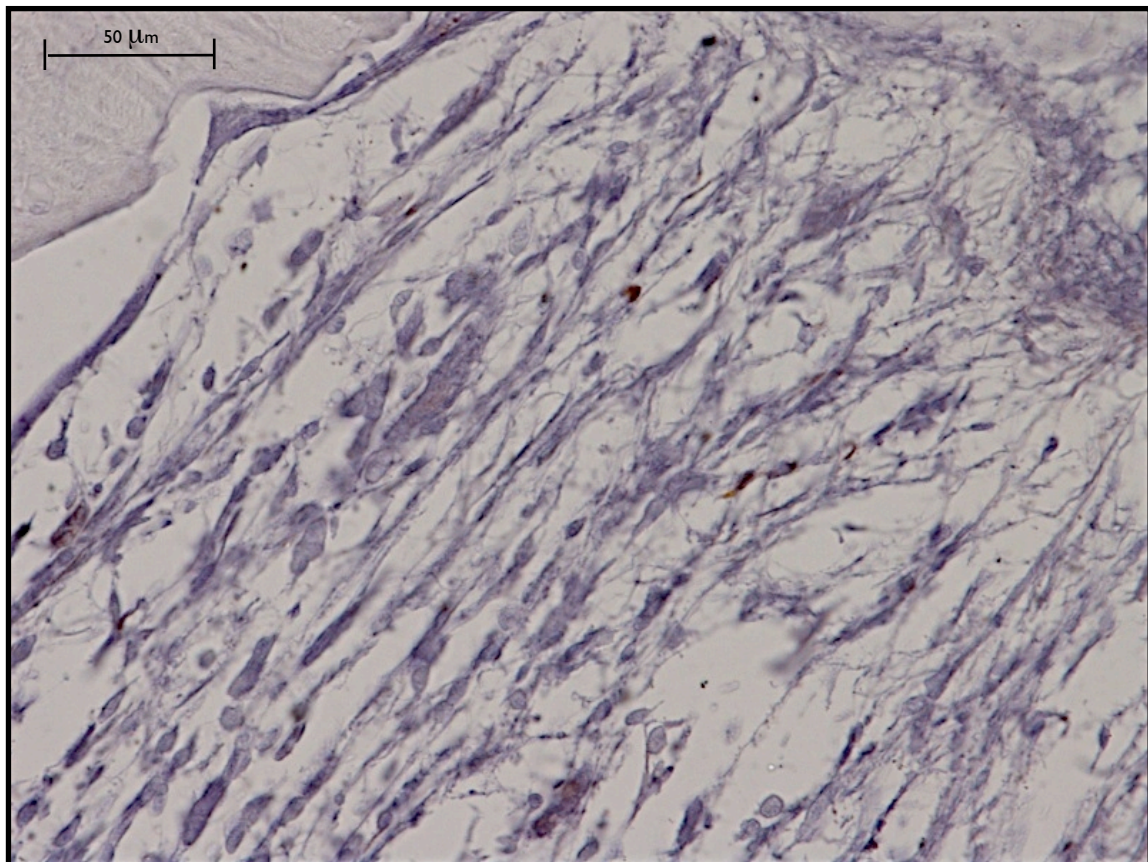Fig.27 IL-6 marking at T0, T1 in test group and control.



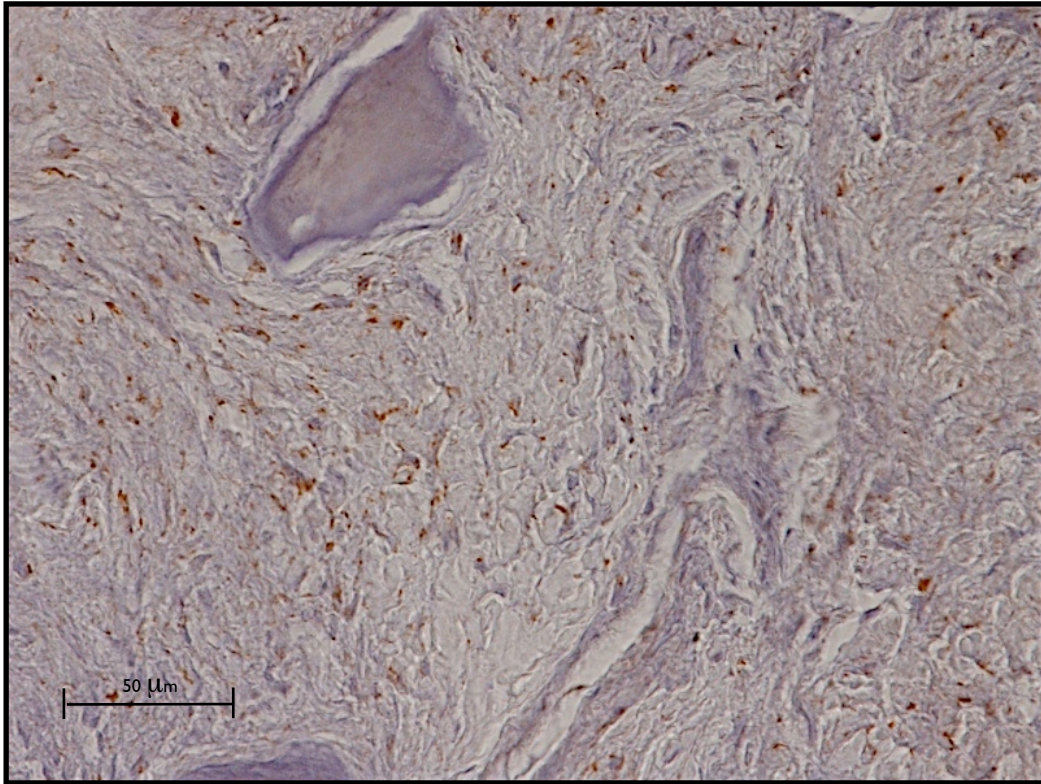Fig.28 IL-6 marking at 200X magnification at T0.

Fig.29 IL-6 marking at T1 in test group at 200X total magnification. It is visible a high marking of IL-6 near DBB particles.
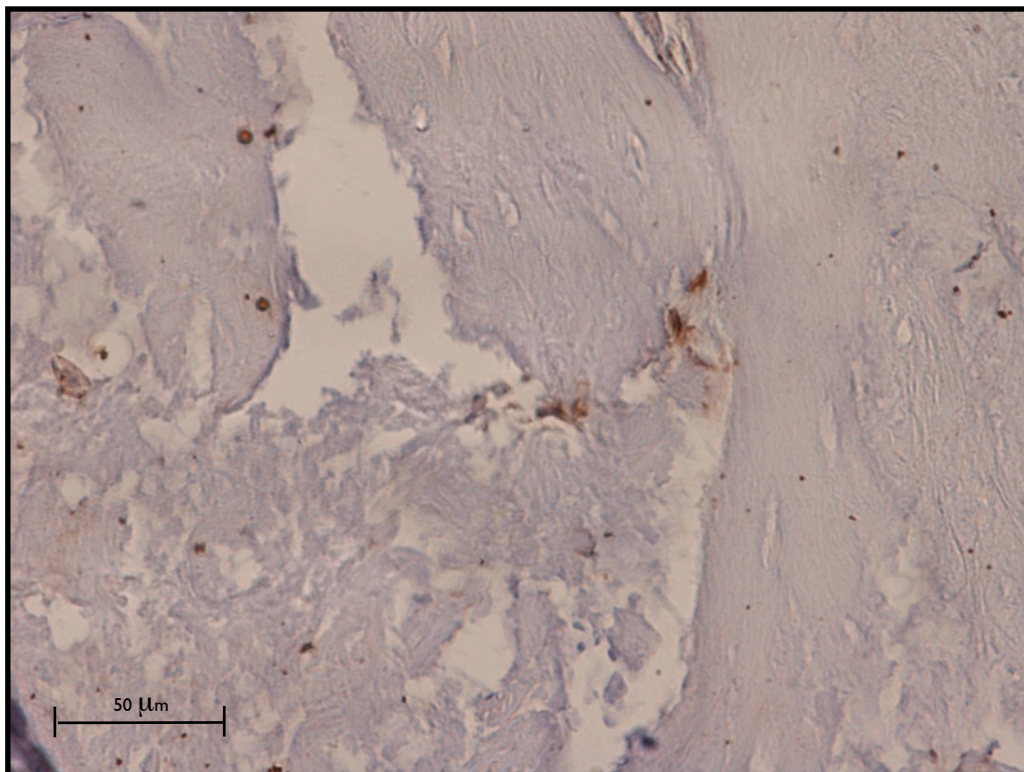


Fig.30 IL-6 marking at T1 in control group at 200X total magnification.

**TNF-α**

In both groups at T0 and T1 similar results were observed, it is visible a slight extracellular staining which partially involved mineralized tissue (Fig. 31-34).
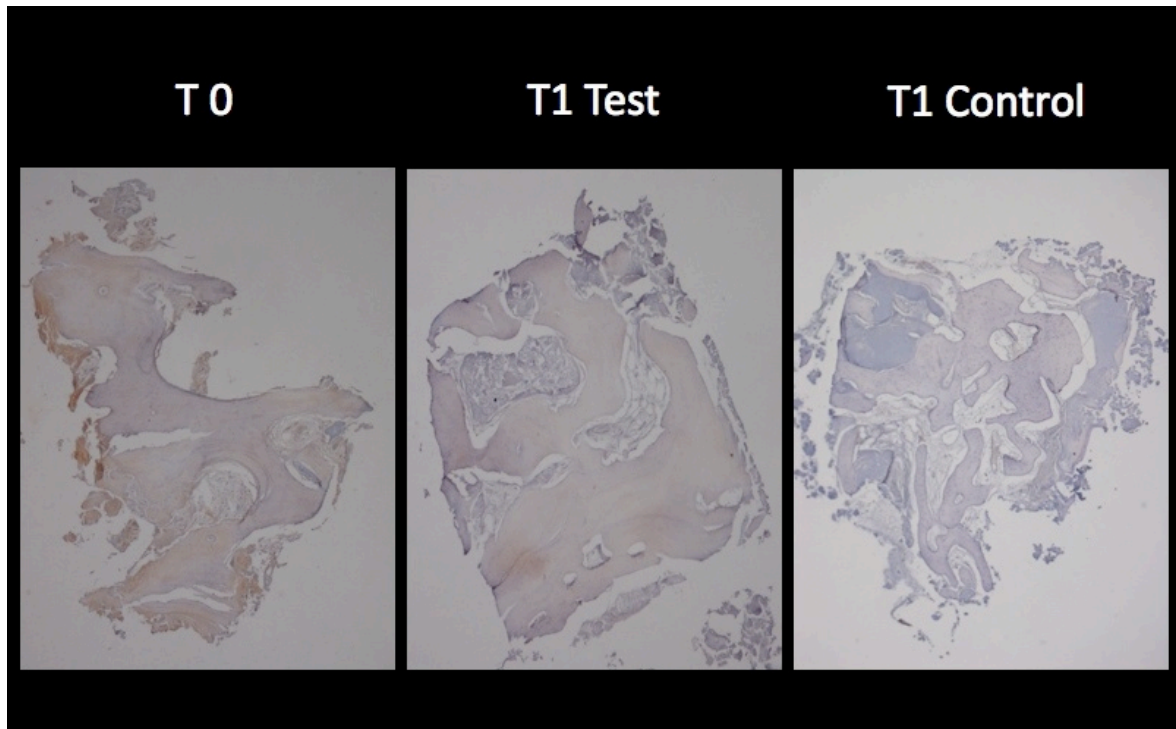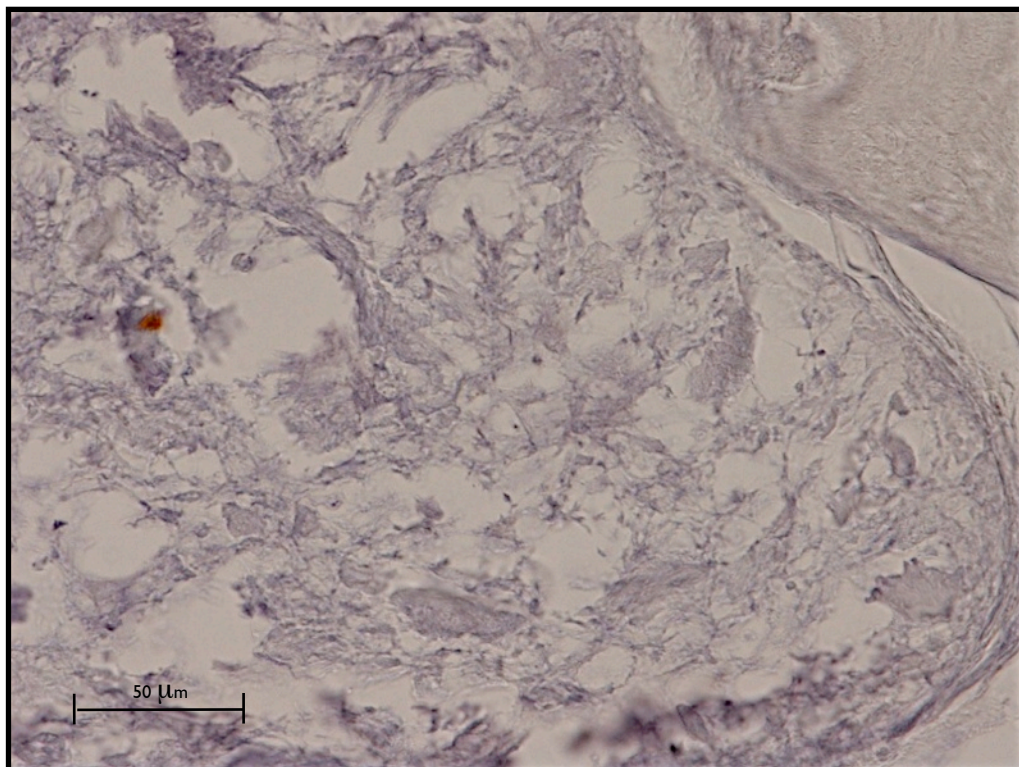


Fig.31 TNF-α marking at T0, T1 test group and T1 control group.
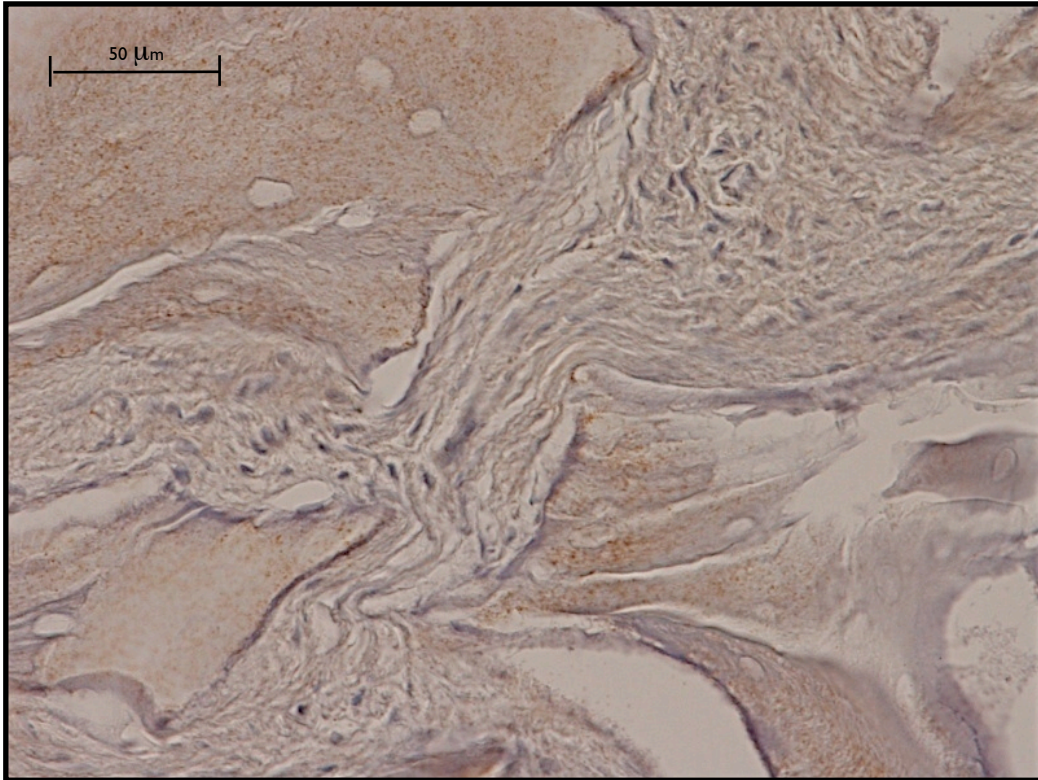


Fig.32 TNF-α marking at 200X total magnification at T0.

Fig. 33 TNF-α marking at 200X total magnification at T1 in test group. It is visible the staining near and on graft particles.
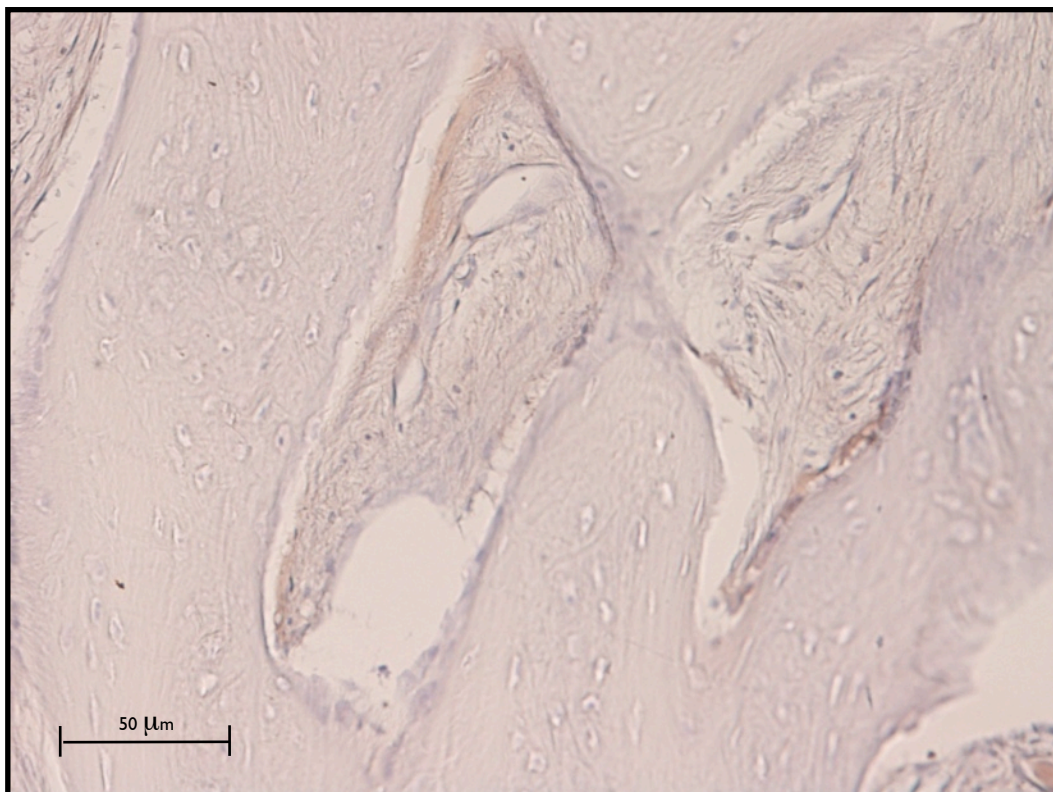


Fig. 34 TNF-α marking at T1 in control group at 200X total magnification.

## 6.5 Statistical analyses

Were found statistically significant differences between  TO and T1 for BMP-2, BMP-7, IL-6 and ALP ($p < 0.05$) for both groups, but in the case of ALP the difference was negative. It was noticed a decrease of ALP values both for test and control. Regarding TNF-α it was found a statistically significant increase from T0 to T1 in test group ($p < 0.05$), while no differences were observed in control group.

The test and control groups were also compared at T1 for every marker; a statistically significant difference was observed in BMP-2 ($p < 0.05$) and TNF-α ($p < 0.05$), while no differences were found for other markers.

**7. Discussion**

The histomorphometric analysis reveals a high percentage of DBB particles still remaining in site at 5 months, these data are in accordance with literature (Cordaro et al. 2008) and confirm that the response obtained in our samples is comparable to other previous studies cited in "rationale" paragraph. Despite the high presence of DBB remained in site, a process of osteointegration was evident, new woven bone was observed surrounding grafts, with bridge of newly formed bone between different DBB particles. The high presence of bone marrow surrounding graft leads to understand that at 5 months the process of new bone formation and osteointegration is still in act in an early phase. This concept is strengthen by the different results observed in the control group. In the control group a higher presence of lamellar bone was found and this verification leads to evaluate the test group in a slightly delayed bone remodeling process. From a clinical point of view, this little delay is balanced by several clinical advantages related to DBB, in particular space maintaining ability as what described in cited literatures. Despite the smaller presence of lamellar bone in test group, the histomorphometric data proved the good osteointegration of graft material and the absence of negative reaction or high inflammatory response.

Shifting from histomorphometric data to immunohistochemistry, we will analyze all the studied markers to better understand the effect of DBB presence on bone remodeling pattern. The observed data are in accordance with literature (Lalani et al., 2003) and show a little increase in both bone morphogenetic proteins from T0 to T1 in both groups. Lalani observed in animal models, that in the first stages of healing BMP-2 were mainly expressed in extracellular matrix of mesenchymal cells, while in later phases, after osteoblast migration and activation, they resulted expressed inside the cells, and in particular inside the osteoblasts. We know, as previously described in the introduction, that animal models may introduce bias, especially on the timing of bone remodeling which is accelerated. For this reason we consider that the data exposed by Lalani et al. may contribute to describe the contemporary presence of extracellular and intracellular expression of BMP-2 at 5 months both in control and test group (Fig.35,36).
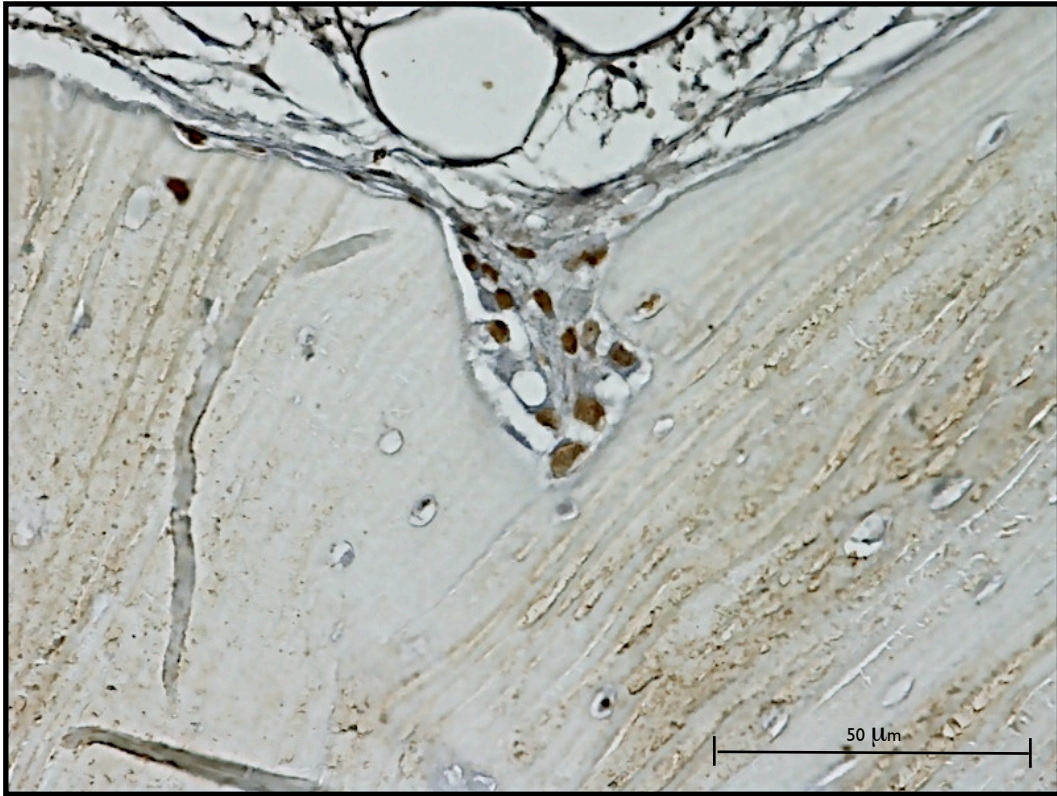
Fig.35 Intracellular BMP-2 marking at T1 in test group at 400x total magnification.
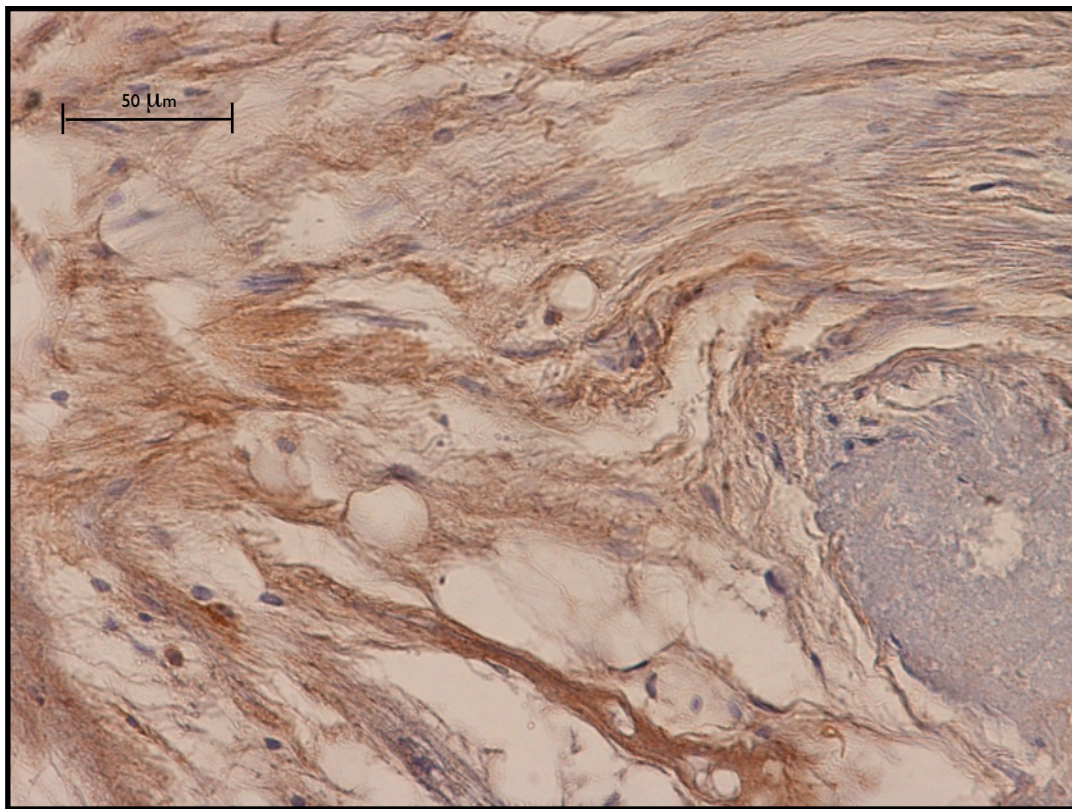


Fig.36 Extracellular expression of BMP-2 in T1 in control group at 200X total magnification.

The results observed both in semiquantitative analysis and in qualitative evaluation are in according with Amerio et al. 2010; we noticed a little increase of BMP-2 marking in both groups, but when comparing normalized difference between test and control we found less BMP-2 expression in test group (p<0.05). In our case we evaluated in vivo in human BMP-2 expression, while Amerio et al. tested the results in an in vitro model that is not able to reproduce the complexity of the event taking part in bone remodeling process, however the data are comparable. This result anyway encourages the use of this graft material when it is necessary for clinical reasons, thus because even we found differences between test and control, in both groups it was found a qualitative increase between T0 and T1 in BMP-2 expression.

BMP-7 expression follows the same increase observed in BMP-2, but it shows a different localization. The increased expression is in accordance with Trombelli et al. (2008); in this case the author performed an in vivo set up and for this reasons we observed the same results. The presence of the grafts, according to our results, is not able to induce a higher proliferation of BMP-7 than what observed in control group (+ 0.36% in test and + 0.10% in control), but the same expression in both groups confirms the efficacy of this material because it combines clinical benefits with the absence of negative effect in term of bone dynamics.

Regarding alkaline phosphatase the observed results are in countertrend with other factors, in both groups it is observed a decreasing in expression value of the semiquantitative analysis. These data are in accordance with what expressed by Thurani et al. (2005) who noticed a decrease expression of ALP in osteoblast in contact with DBB. Furthermore, as what observed by Araújo et al. (2008), there is a little delay in alveolar socket healed with the presence of DBB, but, in these specific cases, the reduced expression is observed also in control group.

IL-6 presented similar results both in test and control group, it was noticed an increasing in both cases between T0 and T1, with no difference between test and control at 5 months. This results explained clearly how at 5 months after extraction the bone remodeling process was in act, with remodeling cytokines expression, stimulating bone resorption.

Concerning TNF-α the results are different between test and control group. In the test group the marking of TNF-α is increased at 5 months, while in control group the marking remained the same from T0 to T1, these results are statistically significant (p<0.05). These data underline the effect of the biomaterial on bone remodeling; the biomaterial is recognized by host cells as a material that needs to be remodeled. For this reason TNF-α is produced by macrophages to stimulate bone resorption and activates osteoclasts cells, while in control group host cells do not activate this process because there is no need to absorb grafts. Anyway, the presence of TNF-α is limited also in test group confirming the ability of DBB of being well tolerated by the organism.

## 8. Conclusions

These results lead to affirm that DBB is able to maintain the activity of all remodeling process. On the other hand it brings to a lower expression of BMP2 and a higher expression of TNF-α compared to what expressed in a similar model healed spontaneously. It is the clinician who needs to evaluate if the reduction of BMP-2 and the increasing of TNF-α evaluated at 5 months, compared to control group, is balanced by the clinical needs for scaffold effects or not. When scaffold effect is required to maintain bone dimension before implant placement, and the clinical efficacy of grafts for this goal is proved by scientific literature, as in the case of DBB, the use of a graft is suggested. Although we noticed differences at 5 months between test and control in BMP-2 expression, in both groups it was observed a significant increasing ($p < 0.05$) between T0 and T1. These results help us to observe that spontaneous healing brings to higher expression of bone morphogenetic proteins than what happens in alveolar socket preservation with DBB, but in both cases the healing process is efficient and this is confirmed by increasing in both groups of BMP expression from T0 to T1. The reduction of ALP expression from T0 to T1 is observed in both groups and it was not found any difference at T1 between the 2 groups. For this reason it can be said that at 5 months the healing site is not completely mature because new bone apposition is required to have complete bone regeneration and we had confirm of this with the positive result found with IL-6 in both groups. Nevertheless the quantity of the bone seen at histological analysis and found by clinicians, is sufficient to correct implant placement and DBB seems to not affect, neither negatively or positively, this result. A different situation was found for TNF-α: in control group it was not found any increasing between T0 and T1, while in test group it was found a statistically significant increasing at T1, with significant results for both tests (T0 vs T1 $p < 0.05$, T1 test vs T1 control $p < 0.05$). The higher expression of TNF-α is justified by the presence of the grafts that need to be remodeled and substituted with new bone. Despite the limited number of the cases involved in this study, it can be affirmed that DBB does not influence negatively alveolar socket healing after tooth extraction and, for this reason, when a graft is required for clinical needs it can be used without any biologic negative interaction. Otherwise, when graft is not strictly useful for clinical benefit, spontaneous healing is suggested because it promotes more BMP-2 expression and leads to a lower TNF-α production.

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