A morphological and immunohistochemical study on the Guided Bone Regeneration Technique (Gbr) with not resorbable membrane

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Abstract

***Introduction*: The success of dental implants largely depends on the quality of osseointegration, a complex biological process regulated by several molecular markers. This study aims to evaluate the expression of three key osteogenic biomarkers—Runx2, osteopontin (OPN), and osteocalcin (OCN)—during bone regeneration in critical-size defects treated with biomaterials. *Materials and Methods*: Critical bone defects were surgically created in rabbit calvariae and filled with various bone substitute materials. Samples were collected at defined healing intervals. Histological, histomorphometric, and immunohistochemical analyses were performed to assess Runx2, OPN, and OCN expression patterns and their association with the newly formed bone tissue. *Results*: Runx2 was predominantly expressed in early healing phases, indicating active osteoblastic differentiation. OPN showed strong localization in the mineralization fronts, while OCN expression increased in later phases, correlating with bone matrix maturation. Differences in biomarker expression were observed depending on the type of graft material used. *Discussion*: The temporal and spatial expression patterns of Runx2, OPN, and OCN confirm their pivotal role in different stages of osteogenesis. These findings suggest that monitoring these biomarkers can provide valuable insights into bone substitutes' biological behavior and the osseointegration quality. *Conclusion*: The differential expression of osteogenic markers in response to bone substitutes offers a valuable tool for evaluating the regenerative potential of biomaterials in implant dentistry. Further research is recommended to validate these results in clinical settings.**

Keywords: Osseointegration; osteogenic markers; runx2; osteopontin (OPN); osteocalcin (OCN); bone regeneration; critical-size defect; bone substitutes; immunohistochemistry; biomaterials in implant dentistry.

Introduction

Volumetric alterations of both the maxillary and mandibular bone, with consequent limited possibility of implant-prosthetic rehabilitation, represents a critical consequence of teeth loss (1). Six months after teeth loss, a reduction in both horizontal (29 to 63%) and vertical (11 to 22%) bone volume is generally observed (2). The rehabilitation of patients suffering from bone atrophy represents one of the significant challenges of

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modern implantology. Although the integrity of the jaw bone is preserved through the stimulus of chewing, tooth loss caused by disease or trauma leads to resorption of the alveolar bone (3). Different types of techniques have been proposed to overcome this type of problem, in particular the guided bone regeneration (GBR) technique. Guided bone regeneration (GBR) is a surgical bone grafting procedure that involves the use of membranes that block soft tissue invasion. The membranes are used in association or not with specific filling materials, which aid the regeneration of the bone tissue (4). The GBR technique has the advantage of promoting alveolar bone gain with predictable and stable results (5). This technique is used to facilitate the insertion of endosseous implants in an adequate bone volume and an optimal position. GBR involves the use of a mechanical barrier capable of isolating the surgical site from both epithelial cells and connective tissue to allow the proliferation of osteogenic cells and the formation of new bone (6). To date, different types of membranes have been developed, both resorbable and non-resorbable. Among the reabsorbable ones we remember: collagen membranes; Membranes in polylactic acid esters and citric acid esters; membranes in polylactic acid (PLA) and polyglycolic acid (PGA); membranes in lactic acid copolymers; membranes in glycolic acid and trimethylene carbonate. Among those that are not absorbable, we remember membranes in e-PTFE, membranes in Titanium reinforced d-PTFE and membranes in titanium mesh. Non-absorbable titanium-reinforced limbs are considered the gold standard for vertical and horizontal ridge augmentation (7).

The state of the art suggests that biocompatibility is the fundamental requirement for a membrane to allow correct bone regeneration. This characteristic enables correct healing by preventing connective tissue growth (8).

Over the years, results suggest that the GBR procedure is a predictable method. In this regard, we can observe a significant variability in the studies that depends on the materials used, the operator's experience, and the type and number of patients chosen. As with membranes, there are also different classifications for the grafting material. Overall, we distinguish the graft material as reabsorbable, non-resorbable, and autologous or non-autologous. The main characteristics of autologous bone are osteoconductivity, osteogenicity, and osteoinduction.

Furthermore, autologous bone contains growth factors in its matrix that exert osteoinductive activity and is currently considered the best graft material (9). Although considered the gold standard for bone regeneration procedures, other filler materials have been investigated to minimize morbidity associated with the donor surgical site (10). There is a large variety of grafting materials on the market. In detail, the graft material must have structural characteristics that simulate the structure of autologous bone, primarily porosity. Since these materials are only osteoconductive and non-osteoinductive, porosity is paramount.

The bibliography shows that the granulometry for correct neo-giogenesis and transformation of our graft must include granules with a diameter between 200-400 microns. A smaller granulometry does not allow correct neo-angiogenesis in the graft (9). In particular, in large grafts where the percentage of bio-material is greater than that of autologous bone, an area with a longer healing time is highlighted compared to cases with a lower quantity of graft material (11). Currently, several types of materials can be used for bone regeneration. Among the main ones we remember are autologous bone, from patient sampling; heterologous bone of animal origin; alloplastic materials, and biomaterials with osteoconductive properties.

In this study, a combined horizontal and vertical GBR technique was performed. The graft was a mixture composed of 50% autologous bone (AB) and 50% allograft of bovine bone with termic deantigenation (ABBMT) or enzymatic deantigenation (ABBME). Finally, a titanium-reinforced PTFE membrane stabilized with pins and screws was used, and the mucosal flap was closed free of tension with a PTFE suture. Starting from all these considerations, this study aimed to evaluate the quality of the regenerated bone in terms of histology and immunohistochemical analysis.

Materials and Methods

Seven patients were selected and underwent GBR technique (Table 1). Eight months after surgery, the membranes were removed, and titanium implants were placed. The implant osteotomy was realized with a 3 mm diameter trephine burs, obtaining bone biopsies, then investigated by morphological and immunohistochemical analysis. The ethics committee approved the study design of Unicamillus n. E03828-2020.

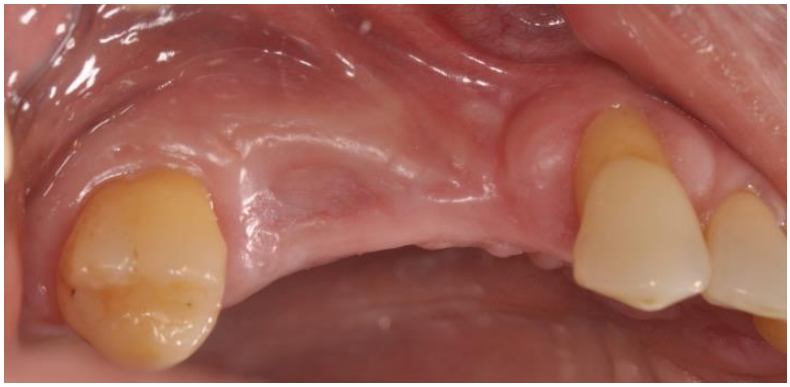

Figure 1. Occlusal view of the defect site 14-15-16 with scaring results.

Table 1. Patient characteristics and data protocol (ABBMT: anorganic bovine bone thermally demineralized; ABBME: anorganic bovine bone enzymatically demineralized; AB: Autologous bone)

Sex	Age	Pathology and pharmacy	Smoke	Position	Types of atrophy	% Osso-Bio	Biomaterial	Membrane	Hematoxylin eosin level 1: trabecular alteration	Hematoxylin eosin level 2: Section 50 um from the previous one. Differences from the first level	Toluidine blue level 1: Bone alterations	Toluidine blue level 2: Section 50 um from the previous one. Differences from the first level	bMP-2 (score 0-3)	BMP-7 (score 0-3)	PTX3 (score 0-3)
F	69	Breast cancer-Etirox e Lobivon	Yes	24-25	Mixed	50-50	ABBME	CYTOPLAST	Yes, by thickness and number. Signs of osteocytes degeneration.	No	Partial loss of firmness. Presence of trabecular rarefaction.	No	0	0	0
F	68	Coarotid stenosis and hypertension	No	26-27	Mixed	50-50	ABBMT	CITOPLAST	No	No	No	No	1	0	1
F	54	Thyroid nodules	No	15-16	Mixed	50-50	ABBMT	CYTOPLAST	No	No	No	No	1	1	0
F	58	Drug allergy	Yes	15-16	Mixed	50-50	ABBMT	CYTOPLAST	Moderate. Slight thinning of the thickness.	No	No	No	1	0	0
M	72	Ndr	Yes	15-16	Mixed	50-50	ABBMT	CYTOPLAST	Yes, by thickness and number. Signs of osteocytic degeneration.	No	No	No	1	1	0
F	63	Allergy to Aulin	No	24-25-26	Mixed	50-50	ABBMT	CYTOPLAST	No	No	Slight	No	0	0	1
F	55	Ndr	Yes	35-36	Mixed	50-50	ABBMT	CYTOPLAST	No	No	No	No	1	2	0

Details on the patients belonging to the study are shown in table 1. In detail, all the cases were treated with the same surgical technique (GBR). 7 patients were selected (1/7 male and 6/7 females), with a median age of 63 years (54-72). Of the selected patients, 4/7 were smokers, and 3/7 were non-smokers. Bone regenerations were performed 6/7 in the maxilla and 1/7 in the mandible. 6/7 cases were treated with 50% autologous bone and 50% thermally deantigenated heterologous bone of bovine origin (ABMMT), 1/7 case was treated with 50% autologous bone and 50% heterologous bone of bovine origin enzymatic deantigen (ABMME).

Surgical Technique

All cases were treated according to the GBR technique. The images below show the different steps of the surgery.

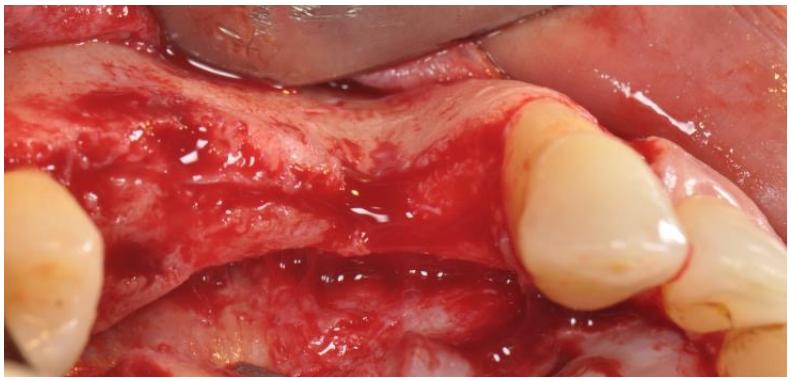

Figure 2. The first step consists of executing a full-thickness safety flap. The technique involves one crest incision and three releasing incisions. The latter are one mesial, one distal, and one palatal. The “hockey stick”-shaped mesial buccal releasing incision begins from the middle of the mesial papilla of the tooth near the defect and ends in the vestibule. The second releasing incision, defined as distal, starts from the most distal point of the crystal incision and continues vestibular into the mucosa. The palatal incision is performed mesial to the tooth close to the defect and extends for approximately 5 mm. In this surgical phase, the vestibular flap is removed through two incisions: the first on the most apical portion of the flap and the second on the most coronal portion of the flap. These incisions allow the flap to be released and allow retention with subsequent lengthening.

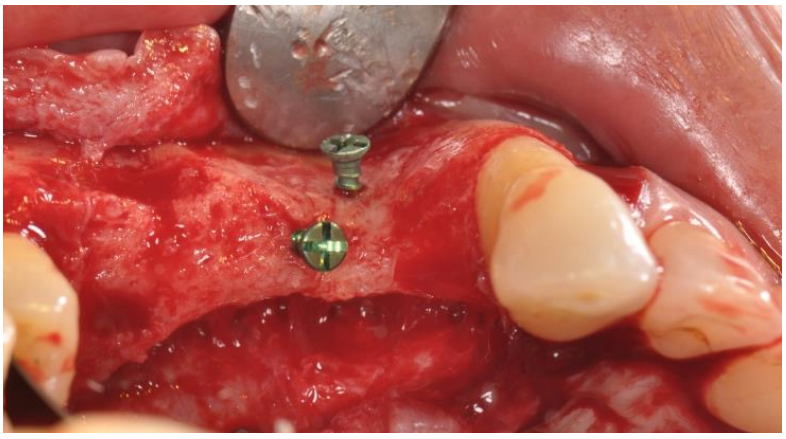

Figure 3. This image highlights the positioning of 2 curtain screws to support the d-PTFE membrane, following the skeletonization of the site to be regenerated.

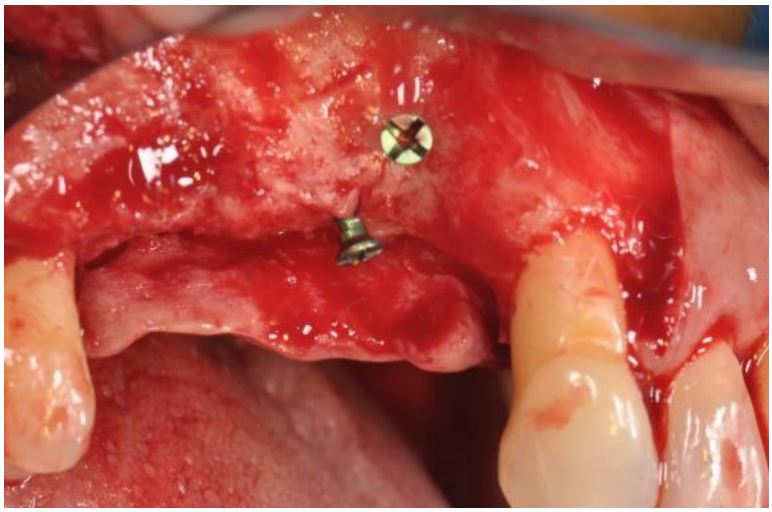

Figure 4. Photo of the preparation of the recipient site with 3 mm drills to promote new angiogenesis.

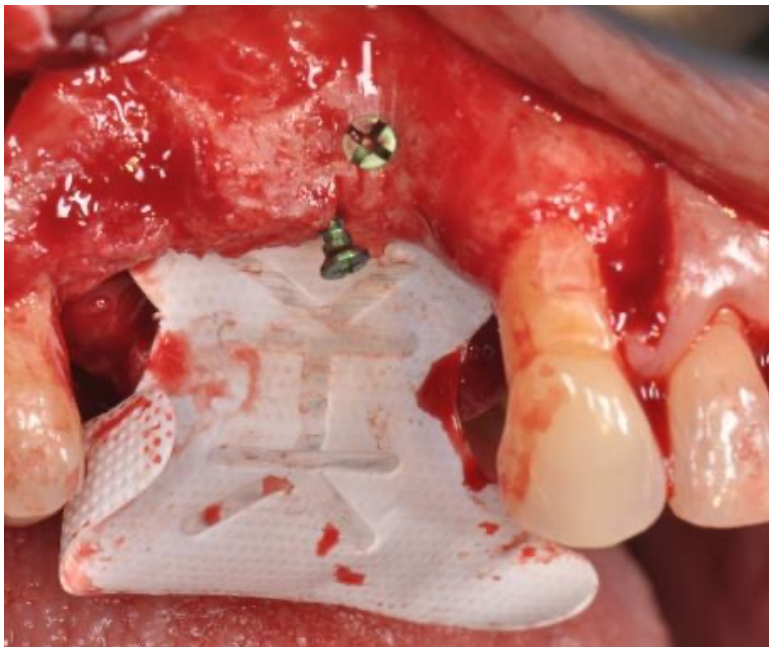

Figure 5. This step highlights the positioning of the membrane reinforced with d-PTFE via two pins on the palatine side of the maxillary bone.

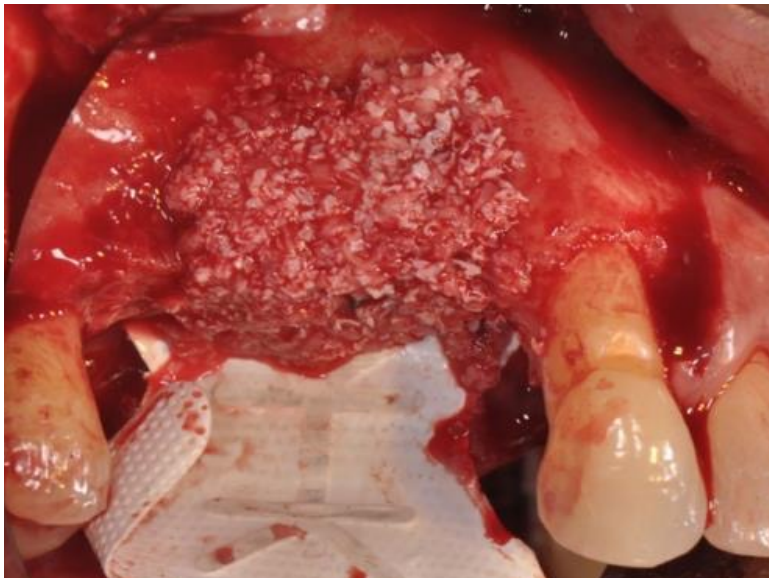

Figure 6. Filling the area to be regenerated with a mix of autologous and bovine bone in a 50%-50% ratio.

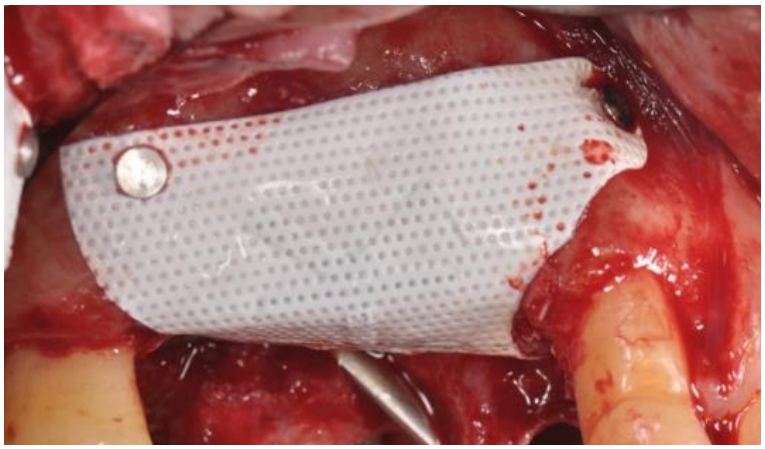

Figure 7. Reversal of the d-PTFE membrane to cover the grafting material. Fixing the membrane with pins

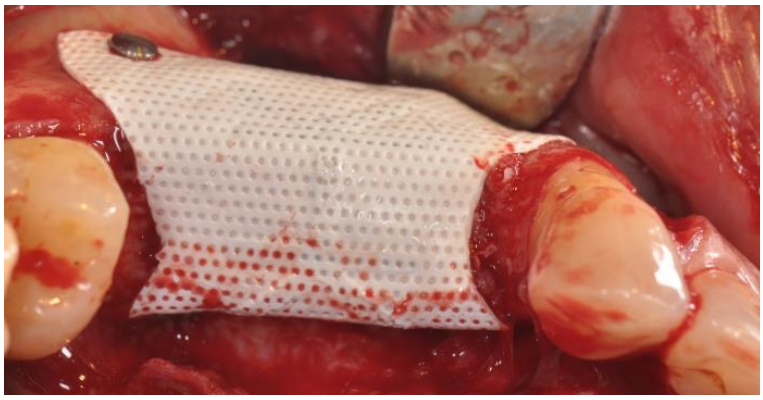

Figure 8. Occlusal view of the defect covered by the membrane where the modeling of the new bone crest is highlighted.

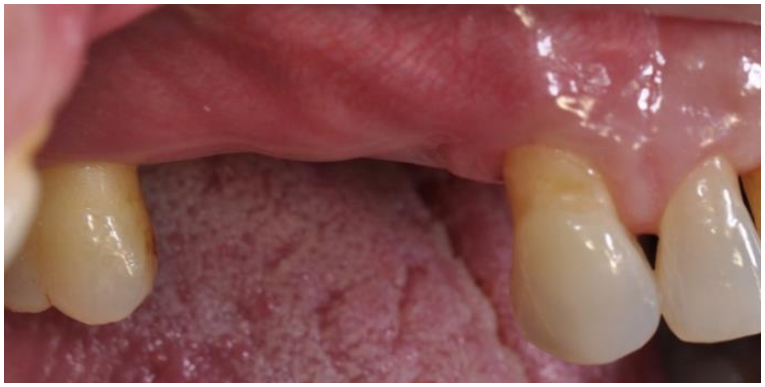

Figure 9. Vestibular view of the defect, which appears to have healed after 9 months.

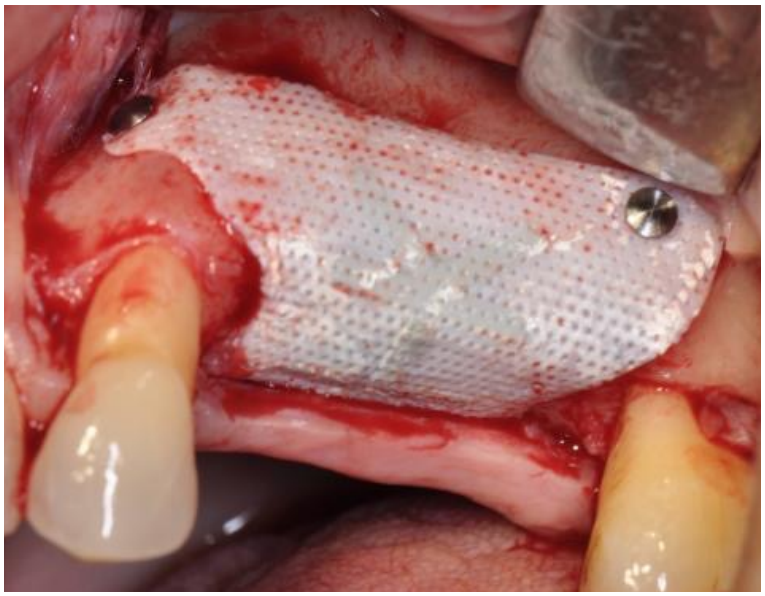

Figure 10. At the end of the 9 months, the regenerated site is reopened to remove the membrane.

Histological analysis

Specimens were fixed in 4% formalin and paraffin-embedded. Four-*µ*m serial sections were used to perform morphological studies (hematoxylin, eosin staining, and toluidine blue staining). Hematoxylin and eosin staining sections were analyzed to evaluate the bone matrix in terms of trabeculae thickness and the presence of bone cells (osteoblasts and osteocytes). The structure of the bone was studied by analysis of toluidine blue staining (12).

Immunohistochemical analysis

Immunohistochemical analysis was performed to study the expression of molecules involved in bone metabolisms, such as BMP-2, BMP-7, and PTX3, in all samples (13). Antigen retrieval was performed on 4-**µ**m-

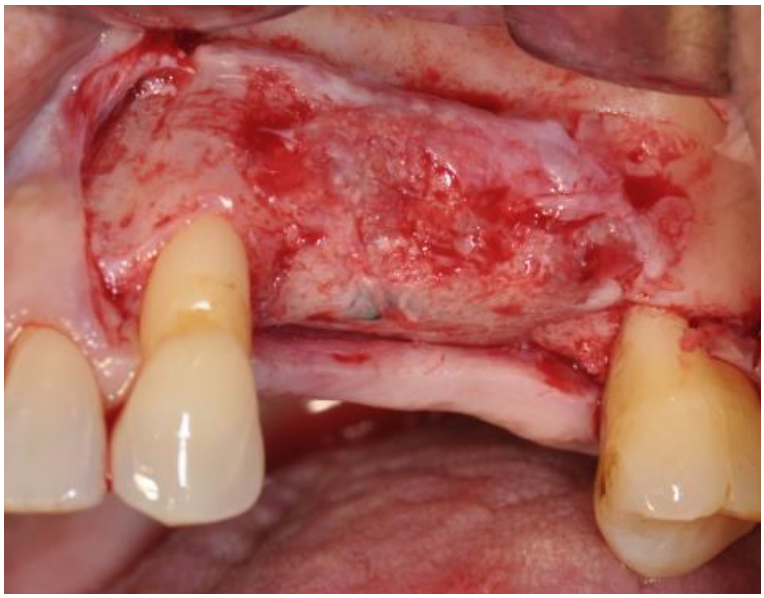

Figure 11. Image of the newly formed bone once the limbs have been removed. The image shows the vestibular and occlusal vision.

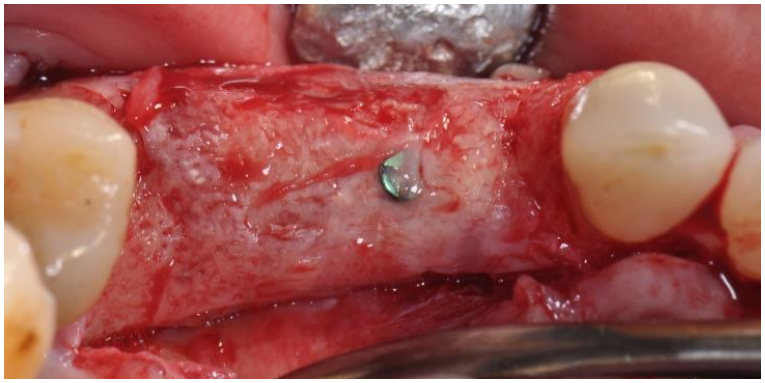

Figure 12. Occlusal image of the regenerated site.

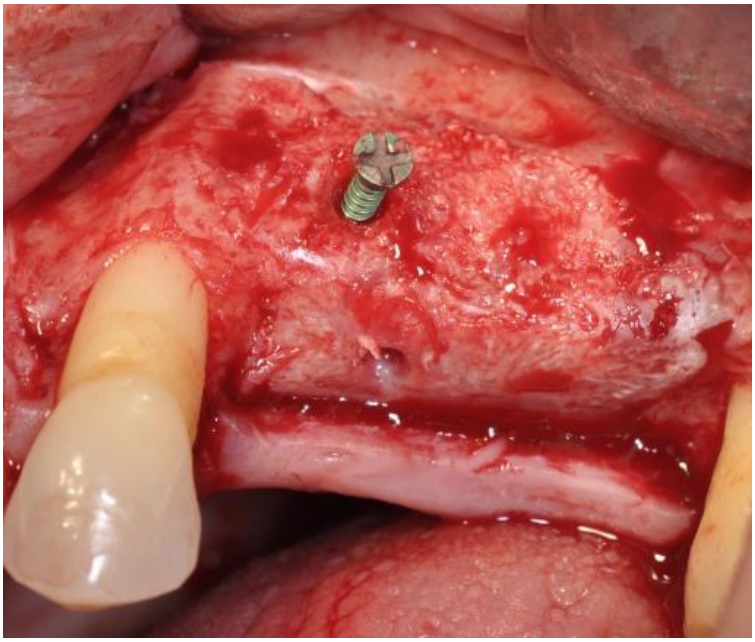

Figure 13. The image highlights the site following the removal of the tent screw of the newly formed bone tissue.

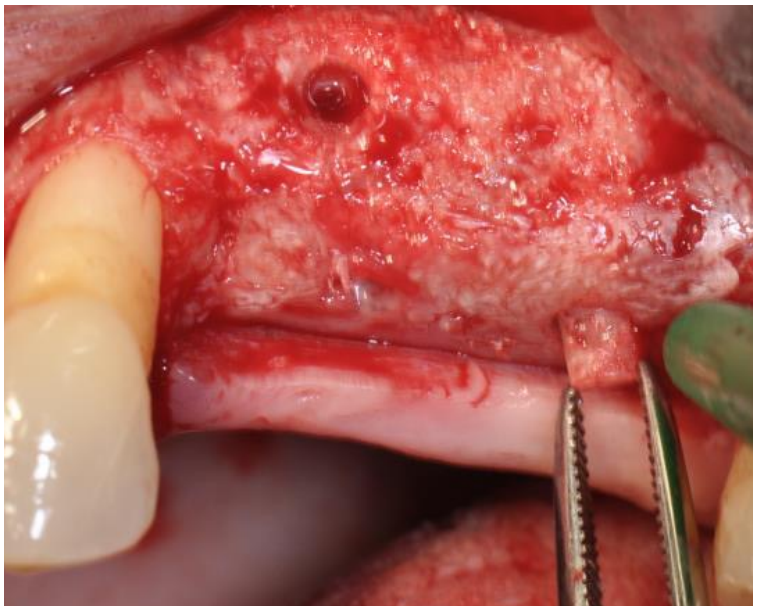

Figure 14. Histological samples were taken using a core drill in this phase. The holes are made for future installations.

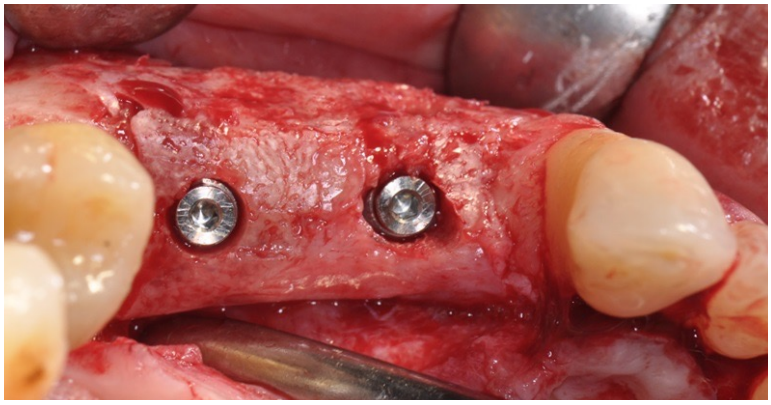

Figure 15. Image of the placement of 2 dental implants.

thick paraffin sections using EDTA citrate buffer pH 7.8 (PTX3) or citrate buffer pH 6.0 (BMP-2 and BMP-7) for 30 min at 95°C. The sections were then incubated for one h at room temperature with the primary antibodies (BMP-2 Mouse monoclonal clone 1A11 Dilution 1:500; Novus Biologicals, Littleton, CO, USA; BMP-7 Mouse monoclonal clone ab54904; Dilution 1:250, AbCam, Cambridge, UK; PTX3 rat monoclonal clone MNB1, Dilution 1:100, AbCam). Washes were performed with PBS/Tween20 pH 7.6. Reactions were revealed by the HRP-DAB detection kit (UCS Diagnostic, Rome, Italy). Immunohistochemical signals were analyzed by assigning a score from 0 to 3 according to the number of positive cells (Table 2) for each section.

Results

Histologic analysis.

Haematoxylin and eosin analysis showed new matrix formation in all biopsies analyzed. Specifically, in 4/7 patients, bone tissues were well formed with neither signs of degeneration nor the presence of an inflammatory infiltrate. In 2/7 patients, the bone

Table 2: Immunohistochemical signals were analyzed by assigning a score from 0 to 3 according to the number of positive cells for each section.

SCORE	Positive cells (10x)
0	No
1	1<x>3 positive cells
2	4<x>6 positive cells
3	>7 positive cells

matrix was well structured, but the new bone was less present than in previous cases. Lastly, less new bone matrix was observed in 1/7 patients. Similar data were obtained by studying the toluidine blue sections.
No difference was observed in the analyzed biopsies regarding the number of osteoblasts and osteocytes. This confirms the presence of a new bone matrix in all patients.

Immunohistochemistry

Immunohistochemical reactions showed an association between the expression of molecules capable of inducing bone matrix deposition and the morphological characteristics of bone specimens. In particular, the highest score values for BMP-2, BMP-7, and PTX3 were observed in patients with higher bone quality regarding both trabecular number and bone thickness. Osteoblasts and osteocytes mainly expressed all these molecules. It is important to note that BMP-2 and PTX3 represent the most potent inducers of osteoblast differentiation and activity, thus contributing to bone regeneration.

Discussion and Conclusions

GBR is a current technique that is still constantly evolving. In particular, according to bibliographical data, autologous bone is still considered the gold standard and the most effective graft material (7).
In this study, we highlighted that in all histological cores (taken in the vertical regeneration component) and with both histological stains (hematoxylin-eosin and toluidine blue), we observed newly formed bone matrix with the absence of inflammatory cells, the presence of vital osteoblasts and osteocytes, and the incremental lines which demonstrate neo-osteogenesis. The regenerative technique was adequate for the regeneration of new bone also in the vertical component. This is in agreement with previous studies (1). The novelty of this study is in the immunohistochemical research related to BMP-2, BMP-7, and PTX3 proteins. We have observed that osteoblasts and osteocytes always express these proteins. Moreover, the BMP-2 and the PTX3 are the most potent osteoblastic differentiation and activity inducers. Their presence suggests the

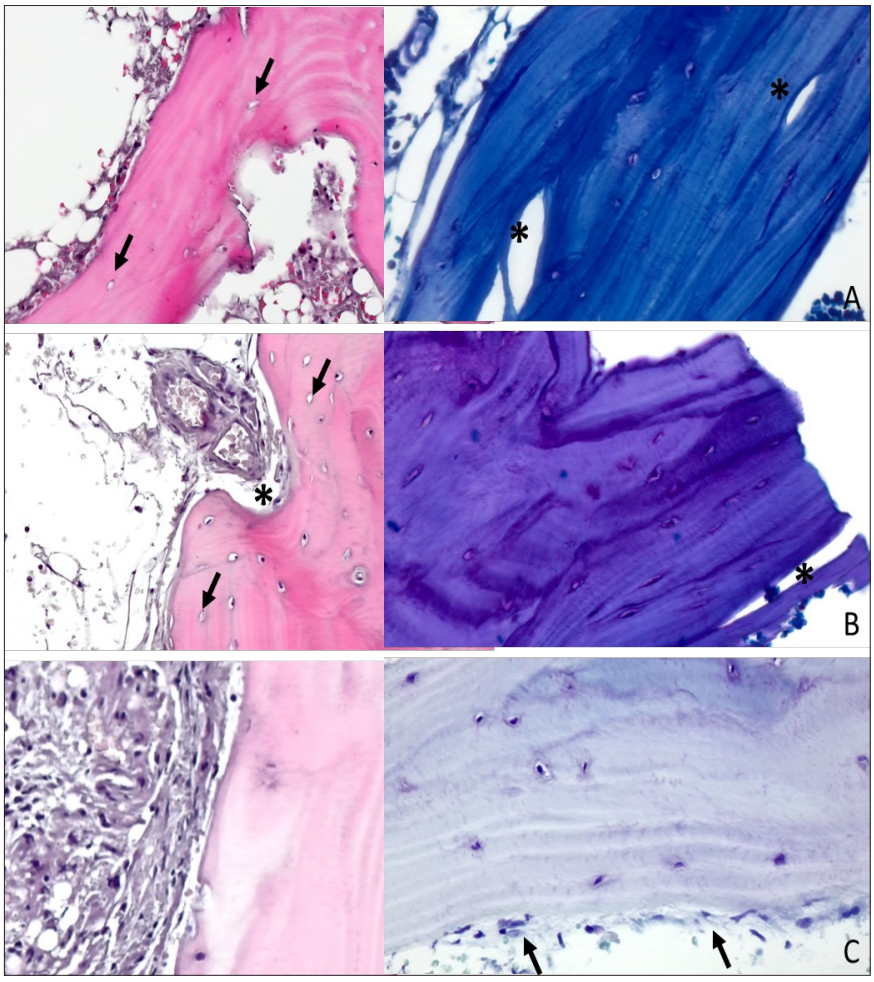

Figure 16. Morphological analysis A) New bone matrix with empty osteocyte lacunae (arrows). B New bone matrix with some osteoblasts (asterisk) and few empty osteocyte lacunae (arrows). C) The image displays well-structured regenerated bone with several osteoblasts (arrows). Magnifications 60x for each image.

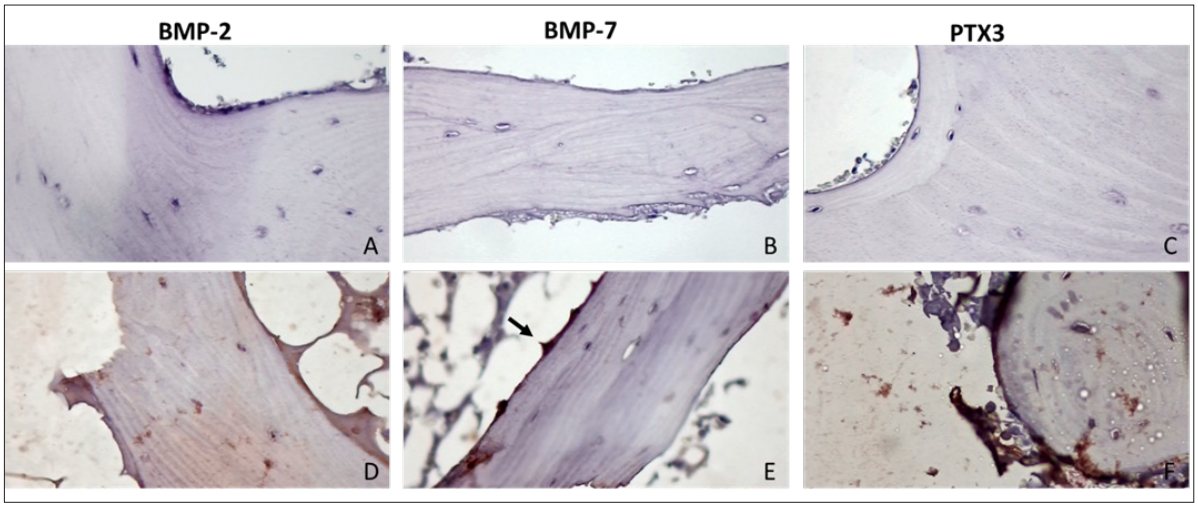

Figure 17. Immunohistochemical analysis. A-C) Regenerated bone with no/rare BMP-2, BMP-7, and PTX3 positive cells is characterized by some empty osteocyte lacunae. D-F) Regenerated bone shows a focal expression of BMP-2, BMP-7, and PTX3. Bone tissue displays numerous osteoblasts (arrows). Magnifications 40x for each image

presence of an active and vital bone capable of driving the osseointegration process and thus supporting the implants. In all samples of the grafted cases autologous bone + thermally de-antigenated bovine bone, we highlighted the presence of these proteins, while in the only sample in which the biomaterial used is enzymatically deantigenated bovine bone, they are absent.

Our study is not the first to perform immunohistochemical analysis. In 2014, the Caballé-Serrano study group carried out the proteomic study of paracrine factors to evaluate their contribution to bone consolidation. The study concluded that proteins released from cortical bone fragments can modulate bone regeneration (15).

In conclusion, the GBR technique with PTFE membranes and an underlying graft of AB and ABBMT can vertically regenerate new bone suitable for supporting implants.

Our conclusions are in line with those in the bibliography. Simion's study group has repeatedly concluded that DBBM and the autologous bone fragment in a 1:1 ratio are an excellent solution for bone regeneration and subsequent dental implant osseointegration (16-17-18-19, 20). The limit of our study is the number of cases examined; future research is needed to confirm our results in a larger cohort.

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