ALGAE-DERIVED HYDROXYAPATITE BEHAVIOR AS BONE BIOMATERIAL IN COMPARISON TO ANORGANIC BOVINE BONE. A SPLIT-MOUTH CLINICAL, RADIOLOGICAL AND HISTOLOGIC RANDOMIZED STUDY IN HUMANS

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Running title: Symbios® Biphasic BGM vs. Geistlich Bio-Oss® Spongiosa in sinus lift.

Key words: Maxillary sinus augmentation; bone biomaterial; anorganic bovine bone; phycogenic biomaterial; morphology; ultrastructure; Implant Dentistry.

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ABSTRACT

Objectives: To analyze a modified biphasic phycogenic biomaterial in comparison with anorganic bovine bone in maxillary sinus floor elevation in humans.

Material and methods: Eight male patients in need of bilateral two-stage sinus floor elevation were consecutively recruited for this randomized split-mouth study. A combination of autogenous cortical bone (ACB, 20%) and anorganic bovine bone (ABB, 80%) (ACB+ABB group) or ACB (20%) and modified biphasic phycogenic material (BP, 80%) (ACB+BP group) were randomly assigned to graft each sinus. Patients were followed up for 6 months post-surgery when bone samples were collected for analysis.

Results: Radiographically, bone height gain was statistically higher in the ACB+ABB vs. the ACB+BP group. While the analysis of the biological compartments showed differences in non-mineralized tissue (39.15±20.97% vs. 65.87±28.59%, ACB+ABB vs. ACB+BP respectively; p=0.018) and remnant biomaterial particles (22.62±17.01% vs. 7.96±8.57%, respectively; p=0.028), the percentage of mineralized tissue (38.23±17.55% vs. 24.14±24.66%, respectively; p=0.398), showed no statistically significant difference. In contrast, ACB+ABB biopsies showed higher Musashi-1 positive cells per mm^2 compared to ACB+BP biopsies (811.49±875.30 vs. 236.90±280.81; p<0.018), where the fusiform cells corresponded mainly with fibroblasts, as demonstrated by ultrastructural analysis.

Conclusion: Both combinations of materials exhibited bone formation after 6 months of healing in the maxillary sinus cavity. However, the combination with biphasic phycogenic biomaterial induced a higher radiographical vertical resorption and graft collapse in comparison with the combination with anorganic bovine bone, possibly due to a higher remodeling of the graft.

INTRODUCTION

Bone biomaterials are used in implant dentistry in common procedures, more and more often. Most biomaterials claim good clinical success and optimal promotion of bone formation. It is well known that different families of biomaterials are available for clinicians. In theory, all of them have different biological properties. Autogenous bone has been extensively used and has become the gold standard. However, some critical limitations, such as limited availability, morbidity and high resorption rates, limit the use of this biomaterial alone. To avoid this, other families of biomaterials have acquired relevance, offering biological properties close to the autogenous bone. The myriad of new biomaterials offers clinical solutions to professionals, however, they are sometimes marketed to dentists with limited or no studies to warrant their clinical validity.

One of these biomaterials has been used since 1988, under the name of Algipore®. It is a naturally occurring hydroxyapatite derived from calcifying maritime algae that can be classified as a phycogenic biomaterial. This biomaterial is presented as a granular biomaterial, with a columnar structure, a particle size between 500–1000 μm and 1000–2000 μm (Klein et al. 2009) and interconnective pores in the range of 5 to 10 μm (Tadic & Epple 2004). It shows good resorbable properties over time with a large surface area for protein binding and amino acid adsorption (Mladenovic et al. 2010). It has been demonstrated to promote bone formation in preclinical studies *in vitro* (Turhani et al. 2005b a) and *in vivo* (Scarano et al. 2012b) as well as in clinical environments (Ewers et al. 2004; Ewers 2005; Iezzi et al. 2012). However, few studies have evaluated this biomaterial in humans and even fewer have evaluated modifications of it in a deep manner. One of these modifications (Symbios® Biphasic BGM, Dentsply IH AB) includes 20% of the constituent material of Algipore® and the remaining 80% is ß-tricalcium phosphate (ß-TCP). Because of the high presence of ß-TCP, the material is claimed to resorb faster while maintaining the volume support necessary for bone regeneration and the rest of the properties of the phycogenic material. Clinical evidence on this biomaterial is limited.

In contrast, anorganic bovine bone (ABB), a xenogenic biomaterial, has been widely used, and the literature has plenty of evidence that supports its clinical use in humans. Particularly, our research group has demonstrated that ABB promotes osteoconduction (Galindo-Moreno et al. 2011), vascularity (Galindo-Moreno et al. 2010b), cell attachment and incorporation (Galindo-Moreno et al. 2014), protein adsorption (Galindo-Moreno et al. 2015), slow resorption rates (Galindo-Moreno et al. 2013) and, ultimately, bone formation (Galindo-Moreno et al. 2010a).

It is not risky to affirm that anorganic bovine bone is the most studied and known biomaterial in dental literature. Thus, ABB is considered as the standard control biomaterial in bone regeneration, particularly in sinus augmentation (Jensen et al. 2016).

Because of this, the aim of this study was to evaluate the performance of a modified biphasic phycogenic biomaterial in humans by analyzing the clinical and radiological behavior, the morphometrical and histological components, the protein expressions related to bone formation, and the analysis of specific markers of mesenchymal stem cells, in comparison with anorganic bovine bone.

MATERIAL AND METHODS

Study population

This randomized split-mouth clinical study was approved by the Ethical Committee for Research in Humans by the University of Granada, Spain. All procedures were performed according to the principles of the Declaration of Helsinki. A written informed consent was obtained from every patient before any study procedure was initiated. This study was registered in clinicaltrial.gov with the protocol number NCT03682315. The CONSORT statement was used to guide the reporting of the findings of this study.

Patients were selected from those who were referred to the clinic of the Department of Oral Surgery and Implant Dentistry faculties, of the University of Granada, Spain, to obtain a bilateral maxillary sinus floor elevation. The inclusion criteria were: patients older than 18 and younger than 80 years, totally edentulous or with Kennedy class I in need of a two-stage sinus floor elevation with less than 4 mm of residual bone height in the posterior segments of the maxilla. Subjects with medical problems such as, pregnancy, uncontrolled dental hygiene, active periodontal disease, any kind of sinus pathology or any other disease known to alter bone metabolism were excluded from the study.

Surgical protocol

Each individual took 875/125 milligrams of amoxicillin/clavulanic acid, taking one tablet every 8 hours, starting one day prior to the surgery. This medication was maintained for 7 days. Lateral window access technique according to Galindo-Moreno and co-workers was used to perform each surgery (Galindo-Moreno et al. 2007). A curved bone scraper (Safescraper®, Meta, Reggio Emilia, Italy) was used to expose the Schneiderian membrane and collect maxillary autologous cortical bone (ACB). Because the size of the lateral window plays an

important role in the maturation of the graft (Avila-Ortiz et al. 2012), a modification of the conventional lateral window was introduced in this protocol. The lateral aspect of the window was designed to be as long as the therapeutic area in the mesio-distal extension while, in the vertical dimension, the window was as small as possible, resulting in a very marked quadrangular shape. Thus, all bone biopsies were collected from areas were the lateral window had been minimally removed. All the surgeries were conducted by the same surgeon and assistant (P.G.-M. and L.G.-G.). The area and dimensions of each surgical window was measured and recorded. Immediately after the sinus membrane was raised, the right and left sinuses were randomized to one or the other combination of biomaterials using a list previously generated in a web-based randomization software ⟨http://www.randomization.com⟩. An assistant not participating in the study generated the list and kept it concealed to the surgeon. All examiners and patients were blinded to the treatment assigned to each side. The control side was grafted with scraped autogenous cortical bone (ACB) in combination with 250 to 1000 μm particle size of xenograft anorganic bovine bone (ABB) (Bio-Oss® Spongiosa, Geistlich Pharma AG) in a 20:80 ratio (ACB+ABB group). The test side received a mix of 500–1000 μm and 1000–2000 μm particle size of biphasic phycogenic biomaterial (BP) (Symbios® Biphasic BGM, Dentsply IH AB) combined with ACB in a 20:80 ratio (ACB+BP group). An equal volume of biomaterial composites were used in both sinuses of each patient adding saline to allow appropriate handling of the particulate mix.

In all cases, an absorbable collagen membrane (Bio-Gide®, Geistlich Pharma AG) was placed over the lateral aspect of the bony window after bone grafting to prevent soft tissue invasion. The area was then carefully closed with 3/0 surgical silk (Laboratorio Aragó, Barcelona, Spain) to achieve primary wound closure.

After a 6-month healing period, during the intervention for implant placement, 3.5 x 22 mm trephines (Salvin Dental Specialties, Inc, Charlotte, NC, USA) were used to collect bone biopsies in the same surgical location where implants were to be placed. The bone cores were processed for histologic, histomorphometric and messenger RNA (mRNA) analyses. At least three biopsies were collected from each sinus, one for histological evaluation, another for mRNA and the third for either cell culture and/or TEM analysis.

Data recorded

A clinical examiner (L.L.-C.) used a standardized clinical research form to register the age, gender, presence of concomitant systemic diseases and chronic use of any medication, alcohol

or tobacco (Galindo-Moreno et al. 2005), history of periodontal disease and the class of edentulism of each patient. Edentulism was classified in two categories: 1) Partially edentulous: patients who presented a Kennedy class I, preserving the anterior teeth; 2) Completely edentulous: patients who had no teeth at all.

Radiographic analysis

Cone beam computerized tomography (CBCT) scans (Imaginarium 700 3D module, Finland) were performed before the sinus floor elevation, and 6 months later, before implant placement.

The maxillary sinus width (MSW) at 3 different heights from the floor of the sinus (5, 10 and 15 mm), the residual alveolar crest (RAC) and the vertical bone height (VBH) before and after sinus floor elevation, were measured in three different antero-posterior points of the intervened area (mesial, central and distal), using constant anatomical landmarks as references, by an experienced surgeon (M.P.-M.), with the Invivo 5 (Anatomage Medical; San Jose, CA, USA).

mRNA analysis

For the evaluation of mRNA, bone cores were immediately submerged in Trizol® reagent, and frozen at -80ºC until processing. For the mRNA extraction, the bone cores were first processed in a tissue blender while submerged in Trizol®. Then, total RNA was isolated following conventional protocol with the instructions provided by the manufacturer (Trizol™ Plus RNA Purification kit, Invitrogen, Grand Island, NY). Total RNA concentration was then measured in a Nanodrop instrument. PrimeScript RT Master Mix (Takara Bio Europe, Saint-Germain-en-Laye, France) was used to generate a final volume of 30 µl of cDNA with 1 µg of RNA from each sample according to the manufacturer's instructions. Finally, quantitative real-time PCR was performed in triplicate by using SYBR Premix Ex Taq II (Takara Bio Inc.), and 2 µl of each sample. The primers analyzed are listed in **Table 1**. The 2^{-ddCt} method was used to calculate gene expression levels relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene commonly used as loading control in RNA studies. All data was normalized to the ACB+ABB group.

Histopathological analysis

For conventional morphology (N.M-M.), as previously reported (Olaechea et al. 2019), bone cores were immediately fixed in 10% buffered formalin for 48 h at room temperature. They were then decalcified for 24 h at 37ºC (Decalcifier I®, Surgipath Europe Ltd., Peterborough, United Kingdom), dehydrated and paraffin-embedded in an automatic tissue processor (Thermo Scientific Excelsior AS, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Biopsies

were later deparaffinized in xylol (3 passes of 5 minutes) and re-hydrated in ethanol of decreasing gradation (absolute, 96%, and 70%, 2 passes of 3 min, respectively). Tissue sections were stained with hematoxylin and eosin, and Masson's trichrome. Apicoronal orientation was determined by considering the areas of the biopsy were no remnant particles were visible as areas of non-grafted native bone. The morphological study (F.O.) was done in a blinded manner on 4-micrometer sections with BX42 light microscopy (Olympus Optical Company, Ltd., Tokyo, Japan), by using the 40x objective in a microscope with an attached scale (BH2, Olympus Optical Company) using a semiquantitative scale (0-3) to assess quantity of bone, non-mineralized tissue, inflammatory infiltrate and remnant particles of biomaterial. The number of osteocytes, osteoblasts, osteoclasts, fibroblast-like cells and vessels were quantified per mm^2. Histomorphometric quantification was performed semiautomatically using the Masson's trichrome stain. The number of images needed to obtain the total extension of the bone biopsy were captured from each sample with a 10x objective in a microscope with a digital camera attached (DP70, Olympus Optical Company), and merged with the Adobe Photoshop CS6 software. Images were then analyzed with the software ImageJ (NIH, http://imagej.nih.gov/ij/) to quantify the percentage of new mineralized tissue, remnant biomaterial particles and non-mineralized tissue in relation to the total area of each part of the biopsy (native and grafted bone). Native and grafted areas were determined by careful evaluation by an experienced pathologist.

Immunohistochemical analysis

As also conducted before (Olaechea et al. 2019), immunohistochemical analysis (N.M.-M.) was done on rehydrated 4-micrometer sections thermally treated in a pre-treatment module (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing a 1mM EDTA buffer (pH 8) at 95ºC for 20 minutes. In order to detect mesenchymal stromal cells (O'Valle et al. 2018), a primary polyclonal antibody against Musashi-1 (MSI1) was then applied and incubated at a 1:100 dilution for 1 h at room temperature. A non-immunospecific IgG was used as a negative control. All antibodies were obtained from Master Diagnóstica (Granada, Spain). The immunostaining was developed in an automatic immunostainer (Autostainer480S, Thermo Fisher Scientific Inc.) using a peroxidase-conjugated micropolymer and diaminobenzidine (Master Diagnóstica).

Posteriorly, both in bone as in non-mineralized tissue, immunopositivity was quantitatively evaluated (F.O.) for osteoblasts, osteoclasts, osteocytes, fibroblasts-like cells and mesenchymal stromal cells per mm^2.

Transmission electron microscopy analysis (TEM)

Several 1-mm² fragments of core graft biopsies were fixed in 2.5% glutaraldehyde solution, decalcified in Decalcifier I® as previously described, and then post-fixed in 1% OsO4 at 4°C for 2 h, washed in distilled water, dehydrated in increasing concentrations of acetone, and embedded in Epon following conventional protocol. Semithin sections were stained with toluidine blue solution. Ten blocks of each area were sampled. Ultrathin (~70nm-thick) sections were obtained in a Reichert Jung ULTRACUT ultramicrotome (Leica, Wetzlar, Germany) and stained with lead citrate and uranyl acetate. Ultrathin sections were examined under a Zeiss Libra 120 transmission electron microscope and processed using specific software for Windows (Soft Imaging System, Berlin, Germany).

Statistical analysis

IBM SPSS-Windows 20.0 (SPSS Inc., Chicago, IL) was used for the analyses. Results are presented as the mean ± standard deviation for continuous variables and as percentages (frequency) for categorical data. The normal distribution of the variables was evaluated with the Kolmogorov-Smirnov test. A non-parametric Wilcoxon signed rank test for paired data (WSRT) was used to evaluate differences between groups. Results were considered statistically significance when p values were below 0.05.

RESULTS

Clinical variables

The sample consisted of eight male patients with a mean age of 59.13 (from 45 to 66 years old). Three patients were partially edentulous; two had diabetes (controlled with insulin and metformin) and hypercholesterolemia (controlled with simvastatin) while another patient presented hypertension with no medication. Five patients expressed alcohol intake in dosages of up to 10 g/day (Galindo-Moreno et al. 2005), four of whom also smoked. All patients had lost their teeth because of periodontal disease; in fact, four of them had suffered severe periodontitis. Six left sinuses were grafted with ACB+ABB. Neither the length (19.38±2.93 and 18.13±2.59 mm; ACB+ABB and ACB+BP, respectively; p=0.250, WSRT) nor the height (5.63±1.77 and 5.38±2.00 mm; ACB+ABB and ACB+BP, respectively; p>0.999, WSRT) of the surgical lateral windows were statistically different between groups.

Clinically, some differences were noticed between groups at the time of implant placement related to the hardness of the newly formed tissue, the presence of soft tissue surrounding some particles and the integrity of the lateral bone formed where the access window was located.

Radiographic results

As shown in **Figure 1**, considering that similar composite graft volumes of each biomaterial were placed in both sinuses, there was visual evidence of differences between groups after 6 months of healing. Sinuses grafted with ACB+ABB showed a higher maintenance of the grafted volume and higher radiopacity. In contrast, sinuses grafted with ACB+BP usually showed more collapse.

The mean initial remnant alveolar crest was 3.70±1.61, 1.73±0.65, and 3.06±1.28 mm (mesial, central and distal) and 4.34±1.83, 2.51±1.34 and 3.36±1.56 mm (mesial, central and distal) for the ACB+ABB and ACB+BP groups, respectively. Differences between groups were not statistically significant for any of the measurement sites (p=0.313, 0.109 and 0.547, mesial, central and distal, respectively; WSRT). Final vertical bone height was 14.31±2.71, 14.76±3.36 and 12.77±2.94 mm (mesial, central and distal) and 10.28±3.47, 10.38±3.82 and 8.59±2.41 mm (mesial, central and distal) for the ACB+ABB and ACB+BP groups, respectively. In this case, differences were statistically significant for all measurements except on the mesial (p=0.055, 0.039 and 0.008, mesial, central and distal, respectively; WSRT). Similarly, bone height gain was 10.61±3.08, 13.03±3.64 and 9.71±3.70 (mesial, central and distal) and 5.94±3.52, 7.87±4.11 and 5.23±2.84 mm (mesial, central and distal) for the ACB+ABB and ACB+BP groups, respectively. Differences in crestal bone height gain were again statistically significant for all measurements except on the mesial (p=0.078, 0.008 and 0.016, mesial, central and distal, respectively; WSRT). Radiologically, maxillary sinus width (MSW) at 5 mm (11.85±2.06 vs. 11.92±1.98 mm, ACB+ABB and ACB+BP groups, respectively), 10 mm (17.15±2.07 vs. 15.52±2.95 mm, ACB+ABB and ACB+BP groups, respectively) and 15 mm of height (19.02±2.97 vs. 18.59±3.98 mm, ACB+ABB and ACB+BP groups, respectively) from the sinus floor was not statistically different between the groups (p=0.867, 0.219 and 0.383; 5 mm, 10 mm and 15 mm, respectively; WSRT). Radiographic data is summarized in **Figure 2** and **Table 2**.

mRNA results

The comparison between the mRNA expression of the proteins ALP (1.00±0.63 vs. 3.45±4.34), MSI1 (1.00±0.92 vs. 0.99±0.89), MSI2 (1.00±1.05 vs. 1.68±1.14), OSX (1.00±0.56 vs. 1.34±0.74), POSTN (1.00±1.03 vs. 1.77±1.33), RUNX2 (1.00±1.20 vs. 1.02±1.08) and SPARC (1.00±0.84 vs. 0.86±0.69) in grafted bone with either ACB+ABB vs. ACB+BP, respectively, showed no statistically significant differences (p=0.383, 0.966, 0.301, 0.359, 0.577, 0.638 and 0.094, respectively; WSRT) (**Figure 3**).

Histopathological results

The semiquantitative morphological assessment (scored 0 to 3) of the grafted area of biopsies showed significant differences between both biomaterials (**Figure 4**), and difference between the induction of non-mineralized tissue (0.63±0.5 vs. 2.29±0.7; ACB+ABB vs. ACB+BP, respectively; p=0.041, WSRT). The presence of inflammatory infiltrate was mild in 2 of 8 of the ACB+ABB biopsies vs. mild/moderate in 5 of 8 of the ACB+BP biopsies.

In the remnant alveolar crest, the percentage of mineralized and non-mineralized tissue of our series were similar: 54.68% vs. 56.82% and 45.32% vs. 43.18%; ACB+ABB vs. ACB+AP, respectively. However, the morphometric analysis of the grafted areas of the samples showed different percentages of mineralized tissue, remnant particles of biomaterial and non-mineralized tissue, as detailed in **Table 3**. Mean values for new mineralized tissue in ACB+ABB were higher than in ACB+BP, although the comparison was not statistically significant. Nevertheless, regarding, non-mineralized tissue, ACB+BP induced 25% more than ACB+ABB. Moreover, the percentage of BP remnant biomaterial after six months of maturation was marginally significantly reduced. While the ABB particles were frequently surrounded by new formed trabecular bone, the BP particles were mostly in contact with non-mineralized tissue (**Figure 5**).

The study of the cellularity present in the biopsies revealed a higher number of cells per square millimeter in sinuses grafted with ACB+ABB, although the difference was not statistically significant (**Table 4**).

Immunohistochemical results

The immunohistochemical expression of MSI1 in MSCs was detected in 7/8 ACB+ABB and 6/8 ACB+BP biopsies. However, ACB+ABB biopsies showed higher MSI1 positive cells per mm^2 compared to ACB+BP biopsies (811.49±875.30 vs. 236.90±280.81; p<0.018, WSRT) (**Figure 6**).

TEM results

Particularly in the semithin sections and the ultrastructural analysis of biopsies from maxillary sinuses grafted with ACB+ABB there was a mixed population of fibroblasts and mesenchymal stromal cells without a developed rough endoplasmic reticulum or surrounded by extracellular collagen. The osteoprogenitor cells were close to the biomaterial particles and the new formed bone trabeculae (**Figure 7A**). Biopsies from the ACB+BP group showed a fibroblastic population with an abundant extracellular matrix and fibrillar collagen seen both surrounding

the particles and inside the pores of the biomaterial. No osteoprogenitor cells were observed in these biopsies (**Figure 7B**).

DISCUSSION

The purpose of the present study was to evaluate the clinical and histological behavior of a modified biphasic phycogenic biomaterial in humans in comparison with the well-documented biomaterial, anorganic bovine bone.

In this study we have described radiographic and histomorphometrical results in humans for the combination of autogenous cortical bone and the biphasic phycogenic material Symbios® Biphasic BGM, as previous studies had also done in a similar material used alone, Algipore® (Schopper et al. 2003; Wanschitz et al. 2005a; Simunek et al. 2005; Kühl et al. 2010). Additionally, histological data, regarding vascularity and cellularity, immunohistochemical detection of the expression of osteogenic markers and mRNA expression of proteins related to bone activity in humans are also presented for the first time. Regarding anorganic bovine bone, Bio-Oss® Spongiosa, similar data have been reported. This information is comparable with previous studies, in spite of different results obtained from different concentrations of the biomaterial reported in the literature.

Clinically, some differences were noticed between groups at the time of implant placement related to the hardness of the newly formed tissue, the presence of soft tissue surrounding some particles and the integrity of the lateral bone formed where the access window was located. Specifically, the sinuses grafted with ACB+ABB had a higher overall structural integrity. These clinical differences were not due to an important technical factor, which is the size of the access window. The dimensions of the lateral window have been shown to influence the outcomes of sinus lift (Avila-Ortiz et al. 2012). In the current study, no differences between groups were found, partly due to a specific effort to perform as small a window as possible. However, the absence of reliable parameters to measure the aforementioned subjective sensations prevent us from describing them further and conducting any type of statistical analysis. The use of bone scrapers to access the sinus cavity is widely supported in the literature (Galindo-Moreno et al. 2007; Martos Diaz et al. 2007; Caubet et al. 2011; Stacchi et al. 2017). Thus, this technical factor was not of influence either.

Radiologically, with data measured through CBCT, we could clearly observe a higher resorption of the ACB+BP composite in relation with the ACB+ABB composite, given that the average bone gain was significantly higher in the ACB+ABB group [10.61±3.08, 13.03±3.64

and 9.71±3.70 (mesial, central and distal) and 5.94±3.52, 7.87±4.11 and 5.23±2.84 mm (mesial, central and distal) for the ACB+ABB and ACB+BP groups, except on the mesial measurement; p=0.078, 0.008 and 0.016, mesial, central and distal, respectively; WSRT], as shown in **Figure 1**, **Figure 2** and **Table 2**. Not only were there differences in the linear measurements, but also in the gray scale of the images. This could indicate different stages of maturation between the two graft composites. These differences and those related to the histological results are not related to the initial maxillary sinus width because we found no differences between groups in terms of that parameter. Maxillary sinus width is known to influence histological results in a negative way: the wider the sinus, the lower the percentage of new mineralized tissue formation. This has been observed to happen irrespective of the surgical approach, lateral (Avila et al. 2010) or transcrestal (Stacchi et al. 2018).

To understand the aforementioned clinical and radiographical differences, the histomorphometrical, histological, immunohistochemical and mRNA analyses take on important relevance. For instance, the presence of new mineralized tissue is higher (38.23±17.55% vs. 24.14±24.66%) in the samples with Bio-Oss® Spongiosa (ACB+ABB) in comparison with those with Symbios® Biphasic BGM (ACB+BP), although no statistical difference was found; however, the percentage of remnant biomaterial particles of Bio-Oss® is higher (22.62±17.01%) vs. the percentage of remnant particles of Symbios® (7.96±8.57%). It is well known that the persistence of non-resorbed biomaterial particles contributes to the maintenance of the graft dimensions. Thus, a higher resorption rate of the biomaterial would lead to a higher overall graft collapse (Galindo-Moreno et al. 2012).

After applying phycogenic hydroxyapatite in a similar model in humans, and 6 months of healing, Scarano and coworkers showed that the percentage of newly formed bone was 35.2±3.6%, non-mineralized spaces were 35.6±2.3%, and residual grafted material was 37.1±3.8% (Scarano et al. 2012a). This is the highest percentage of mineralized tissue formation using this type of biomaterial found in the literature. The authors relate the differences regarding other studies to individual biological variations. However, Schopper and coworkers showed a mean value of 23.0%±8.3% of new mineralized tissue, a mean value of 33.0±7.8% of remnant biomaterial and 44.0±13.7% of non-mineralized tissue after 7 months of healing (Schopper et al. 2003). Simunek and coworkers reported different percentages of histomorphometric compartments, depending of the time of the biopsy, in a two-stage technique. They reported 10.9±9.5% of mineralized tissue and 32.2±4.5% of remnant particles after 6 months but 30.2±7.0% of mineralized tissue and 17.3±11.5% of remnant phycogenic biomaterial after 15

months (Simunek et al. 2005). Thus, overtime, phycogenic biomaterial tends to induce more new mineralized tissue formation as the graft resorbs. We found 24.14±24.66% of new mineralized tissue, 7.96±8.57% of remnant biomaterial and 65.87±28.59% of non-mineralized tissue after 6 months of graft maturation. This is a similar quantity of new mineralized tissue formation as in Schopper´s manuscript, but a lower percentage of remnant particles of biomaterial. We could explain the differences in the amount of remaining particles by the chemical differences between Algipore® and Symbios® Biphasic BGM. Although similar and from the same family, the biphasic composition of the biomaterial used in the current study may explain these differences, particularly regarding the higher resorption of the ß-TCP phase of the material. We also observed differences in other biomaterials from similar families but with different processing methods (Monje et al. 2017). It is also important to note that the ratio of biomaterial in the composite graft may influence the proportion of the different tissue compartments. In any case, in all of our studies with different proportions, the range of new mineralized tissue is quite similar to that found in the current study (Galindo-Moreno et al. 2010b, 2011, 2018).

It is also very important to discuss the differences related to the non-mineralized component. In our study, this important tissue component is higher than in the previously mentioned manuscripts. This has three possible explanations: 1.- The presence of autogenous bone in our graft composites induces quicker metabolic responses in the area, in terms of graft resorption. 2.- Our composite grafts have a shorter healing time [6 months vs. 7 months (Schopper et al. 2003), or 6, 9, 12 and 15 months (Simunek et al. 2005)], with different moments of maturation of the grafted area. In this sense, Simunek and coworkers reported that the longer the maturation time, the higher the new mineralized tissue and the lower the remnant biomaterial. However, in their case series the quantity of non-mineralized tissue stays stable overtime, around 57 to 58%, although this aspect is not described specifically in their manuscript (Simunek et al. 2005). 3.- A higher inflammatory component, not analyzed in previous manuscripts, but notable in our study, both clinically and histologically, was found in the ACB+BP group (5/8 vs. 2/8 patients in ACB+BP vs. ACB+ABB respectively). Although, in humans, other authors described the absence of inflammatory infiltrate (Schopper et al. 2003; Scarano et al. 2012a) in their samples, and immunological in vitro testing of human peripheral blood cells cultivated in the presence of Algipore® have shown no signs of activated immune response (Wanschitz et al. 2005b), our histological results are evidence of the presence of this inflammatory infiltrate in diverse areas of the samples (**Figure 4B).** 4.- In our study, the mRNA analyses show that there are no

statistically significant differences in the expression of any of the studied genes (**Figure 3**). Although some of them may represent a fibrotic type of response (POSTN), the others clearly represent a controlled osteogenic environment. The absence of differences in this respect is similar to the findings from Caubet and co-workers who found no differences in the expression of several genes after 6 months of healing, although some differences were found when the biopsies were collected before that time (Caubet et al. 2015).

Regarding the cellularity, there were no statistical differences between both grafted areas in terms of any of the analyzed types of cells. However, reparation and consolidation of the grafted area is promoted by mesenchymal stromal cells. Thus, in our study, we analyzed the presence of these cells using MSI1 (O'Valle et al. 2018). Previously, our team had proposed the use of MSI1 as a marker to analyze bone regeneration (Padial-Molina et al. 2015) due to the role of MSI1 in bone cells (O'Valle et al. 2015). In fact, we have detected that MSI1 and RUNX2 follow a similar expression pattern during osteogenic differentiation of MSC derived from the oral cavity (Padial-Molina et al. 2019). MSI1 positive fibroblast-like cells in regenerated areas in humans have been demonstrated in other families of biomaterials, as allografts (Galindo-Moreno et al. 2018) and synthetic materials (Olaechea et al. 2019; Flichy-Fernández et al. 2019). In the present study, we have also found a statistically significantly higher number of MSI1 positive cells per mm^2 in the non-mineralized component of the grafted areas surrounding both combination of biomaterials. Similar findings have been previously reported (Olaechea et al. 2019), which would indicate higher activity in the regenerated bone than in the native bone area. Moreover, we should also note that the combination with anorganic bovine bone induced higher MSI1 positive cells than the combination with biphasic phycogenic biomaterial. We were able to further confirm this by the ultrastructural analysis. As described, biopsies from maxillary sinuses grafted with ACB+ABB showed osteoprogenitor cells close to the biomaterial particles and the new formed bone trabeculae (**Figure 7A**). Biopsies from the ACB+BP group showed a fibroblastic population with an abundant extracellular matrix and fibrillar collagen (**Figure 7B**).

Finally, we should mention that many of the studies referenced above (Schopper et al. 2003; Simunek et al. 2005; Scarano et al. 2012a) do not evaluate the portion of the biopsy that corresponds to the original bone crest. However, this portion of bone plays an important role in initiating and serving as a reservoir for osteogenic cells to migrate into the grafted area. In our case, the percentage of native mineralized and non-mineralized tissue were similar comparing

both groups. Thus, the differences found in the grafted area cannot be attributed to differences in the native bone.

Our study presents some limitations. First, bone maturation in grafted sinuses depends on several clinical variables, such as age, gender, habits or anatomical features of each individual sinus. Besides, trephine cores are obtained where implants are going to be located. This requirement makes it quite difficult to standardize the area to be later analyzed. However, this model is well accepted by the scientific community to study bone behavior in humans and animals, in spite of this drawback. Secondly, the sample size could be considered to be small. However, it is in accordance with many studies published in the literature. On the other hand, although there were no statistical differences in many of the studied variables, the clear discrepancies in terms of graft resorption observed in the CBCT techniques between both biomaterials, urged us not to increase the population size. Finally, as always in studies were multiple clinical, radiographic and laboratory exams are conducted, more potential for affecting reproducibility could be introduced compared to studies analyzing only of aspect of a surgical outcome. To prevent this, our study has been conducted by experienced researchers in each particular field.

CONCLUSIONS

According to the results from the current study and considering the aforementioned limitations, both combinations of materials exhibit bone formation after 6 months of healing in the maxillary sinus cavity. However, the combination with biphasic phycogenic biomaterial induces a higher radiographical vertical resorption and graft collapse in comparison to the combination with anorganic bovine bone.

COMPLIANCE WITH ETHICAL STANDARDS

Funding

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Conflict of Interest

The authors declare that although the biomaterials were donated by Dentsply IH AB and Geistlich Pharma AG companies, no resources or economic support were provided by either company for the development of the present research. Pablo Galindo-Moreno is a frequent speaker for both companies at different events. Regardless, the authors declare no conflict of interest, either directly or indirectly, in any of the products listed in the manuscript.

Ethical aspects

All procedures performed in studies involving data from human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

This clinical study was reviewed and approved by the Ethics Committee for Human Research of the University of Granada, Spain. The protocol was then registered at clinicaltrials.gov and assigned the number NCT03682315.

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TABLES

Table 1: Sequence of each of the primers for the genes evaluated in the current study.

GENE	PRIMER FORWARD	PRIMER REVERSE
Alkaline phosphatase (ALP)	TCCAGGGATAAAGCAGGTCTTG	CTTTCTCTTTCTCTGGCACTAAGG
Musashi-1 (MSI1)	TGAGCAGTTTGGGAAGGTG	TCACACACTTTCTCCACGATG
Musashi-2 (MSI2)	CCCACCATGAGTTAGATTCCAAGAC	TTGTGACCATCTTGGGTTGCG
Osterix (OSX)	CTGCTTGAGGAGGAAGTTCAC	TGCTTTGCCCAGAGTTGTTG
Periostin (POSTN)	TTTCTACTGGAGGTGGAGAAAC	GTGACCTTGGTGACCTCTTC
Runt-related transcription factor 2 (RUNX2)	ACCGTCTTCACAAATCCTCCC	AGCTTCTGTCTGTGCCTTCTG
Secreted protein acidic and rich in cysteine (SPARC) *aka* Osteonectin	AGAACAACACCCCCATGTGCGT	TCCAGGGTGCACTTTGTGGCAA
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	AGCTCATTTCCTGGTATGACAAC	TTACTCCTTGGAGGCCATGTG

Table 2: Radiographical measurements.

		ACB+ABB	Interquartile range	ACB+BP	Interquartile range	p-value*
INITIAL HEIGHT	Mesial	3.70±1.61	3.05	4.34±1.83	3.46	0.313
	Central	1.73±0.65	1.21	2.51±1.34	2.47	0.109
	Distal	3.06±1.28	2.53	3.36±1.56	3.05	0.547
FINAL HEIGHT	Mesial	14.31±2.71	4.33	10.28±3.47	5.99	0.055
	Central	14.76±3.36	5.12	10.38±3.82	7.00	0.039
	Distal	12.77±2.94	4.06	8.59±2.41	4.64	0.008
HEIGHT GAIN	Mesial	10.61±3.08	3.33	5.94±3.52	5.71	0.078
	Central	13.03±3.64	5.42	7.87±4.11	6.55	0.008
	Distal	9.71±3.70	4.59	5.23±2.84	5.31	0.016
MAXILLARY SINUS WIDTH	5 mm	11.85±2.06	2.98	11.92±1.98	3.70	0.867
	10 mm	17.15±2.07	4.06	15.52±2.95	5.98	0.219
	15 mm	19.02±2.97	4.81	18.59±3.98	7.23	0.383

ACB: autogenous cortical bone; ABB: anorganic bovine bone; BP; biphasic phycogenic biomaterial; *: Wilcoxon signed rank test for paired data.

Table 3: Percentual area of the different tissue compartments analyzed in the morphological study. Data expressed as Mean±SD.

	ACB+ABB	Interquartile range	ACB+BP	Interquartile range	p-value*
Mineralized tissue (%)	38.23±17.55	35.65	24.14±24.66	49.38	0.398
Non-mineralized tissue (%)	39.15±20.97	41.80	65.87±28.59	53.73	0.018
Remnant biomaterial (%)	22.62±17.01	37.40	7.96±8.57	10.77	0.028

ACB: autogenous cortical bone; ABB: anorganic bovine bone; BP; biphasic phycogenic biomaterial; *: Wilcoxon signed rank test for paired data.

Table 4: Number of cells per mm^2 and vessels. Data expressed as Mean±SD.

	ACB+ABB	Interquartile range	ACB+BP	Interquartile range	p-value*
Osteocytes	148.38±43.19	56.46	185.48±100.72	185.49	0.500
Osteoblasts	48.38±48.00	96.78	19.35±28.85	48.39	0.357
Osteoclasts	4.03±11.40	0.00	3.22±7.21	8.07	0.317
MSI1 positive cells	811.49±875.30	490.32	236.90±280.81	204.77	0.018
Vessels	48.38±37.57	88.71	35.48±33.05	64.52	0.892

ACB: autogenous cortical bone; ABB: anorganic bovine bone; BP; biphasic phycogenic biomaterial; *: Wilcoxon signed rank test for paired data.

FIGURE LEGENDS

Figure 1: CBCT representative image of the visual differences between groups after 6 months of healing. As it can be observed, the left sinus, grafted with ACB+ABB, shows a higher volume maintenance and higher radiopacity after 6 months of healing.

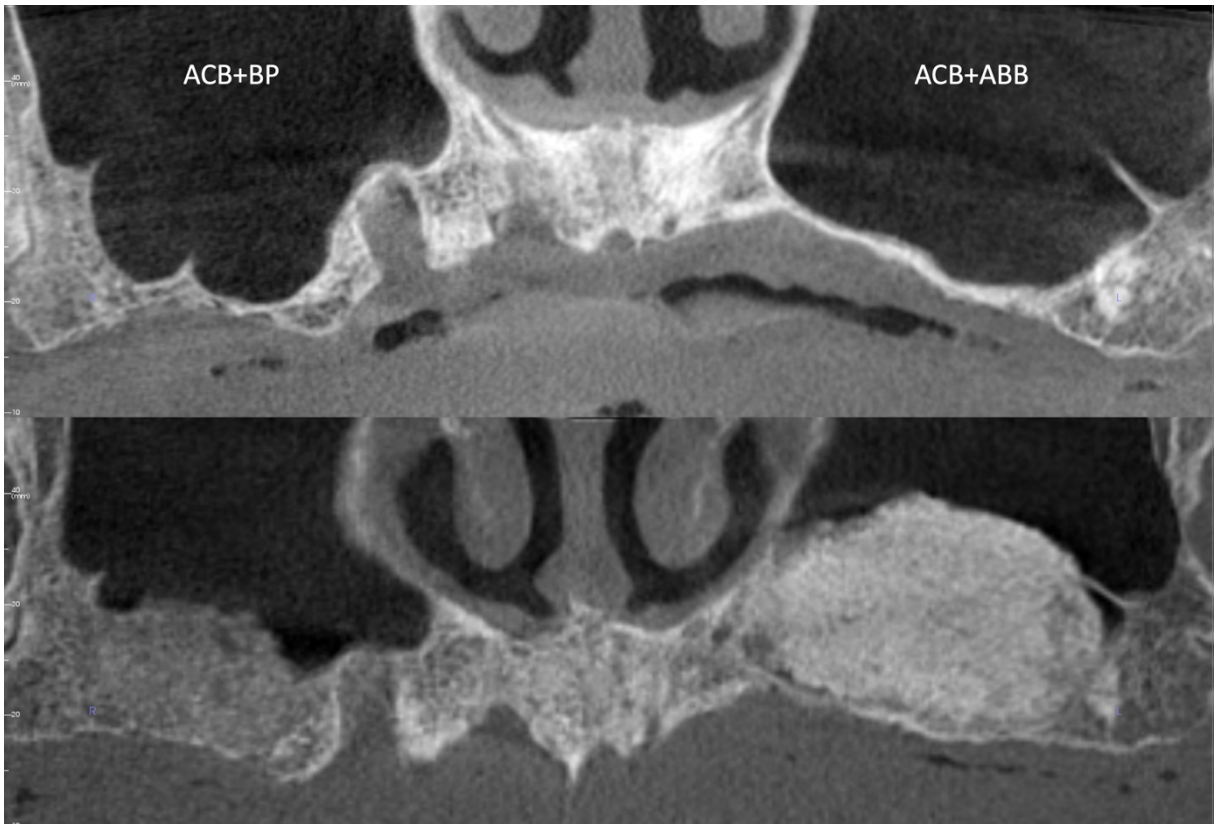

Figure 2: A) Initial (semi-transparent lines) and final (hard lines) crestal bone height for the ACB+ABB and ACB+BP groups at the different antero-posterior position. B) Crestal bone height gain at each antero-posterior position. C) Size of the maxillary sinus access window considering the height and the antero-posterior length. D) Internal bucco-lingual width measured at 5, 10 and 15 mm from the floor of the sinus. Statistically significant differences are denoted with an asterisk.

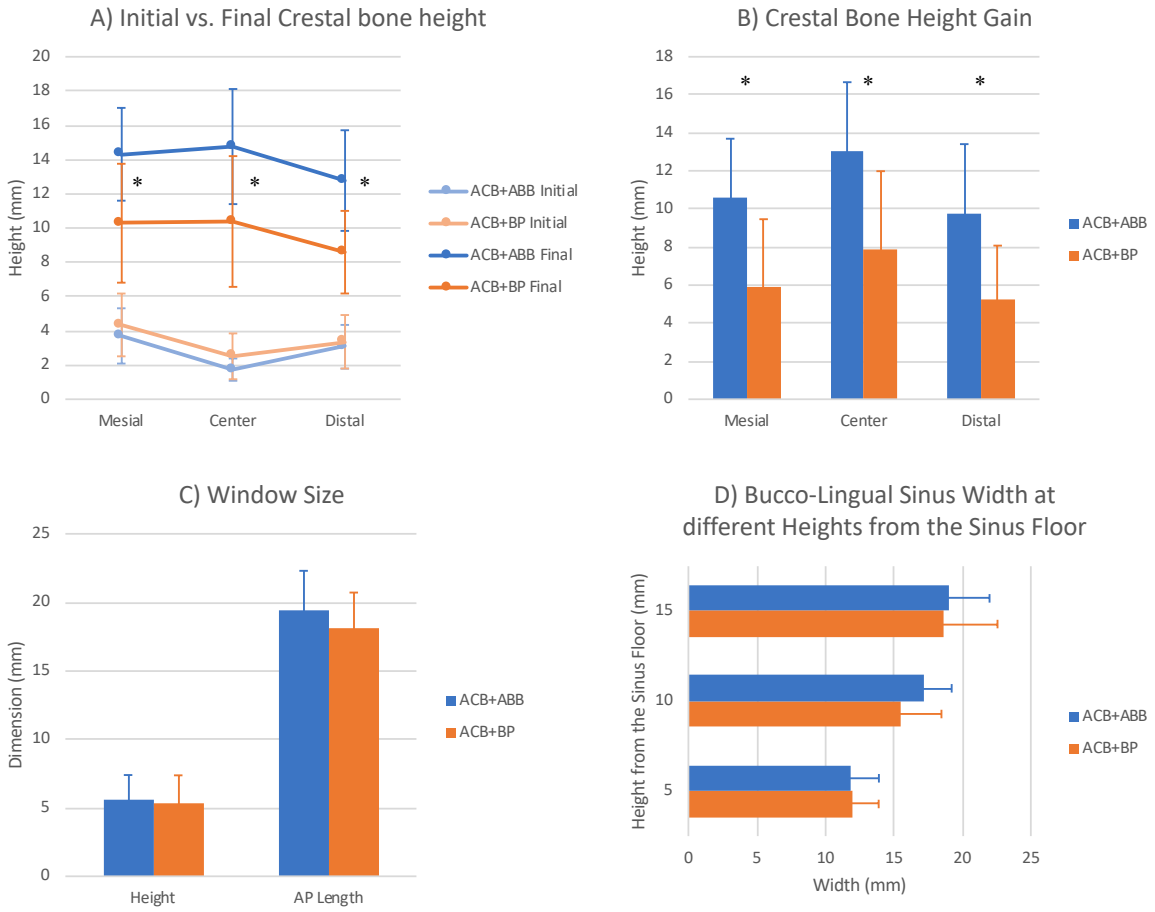

Figure 3: Bar graph representing mean and SD of mRNA expression of different genes relative to ACB+ABB (blue).

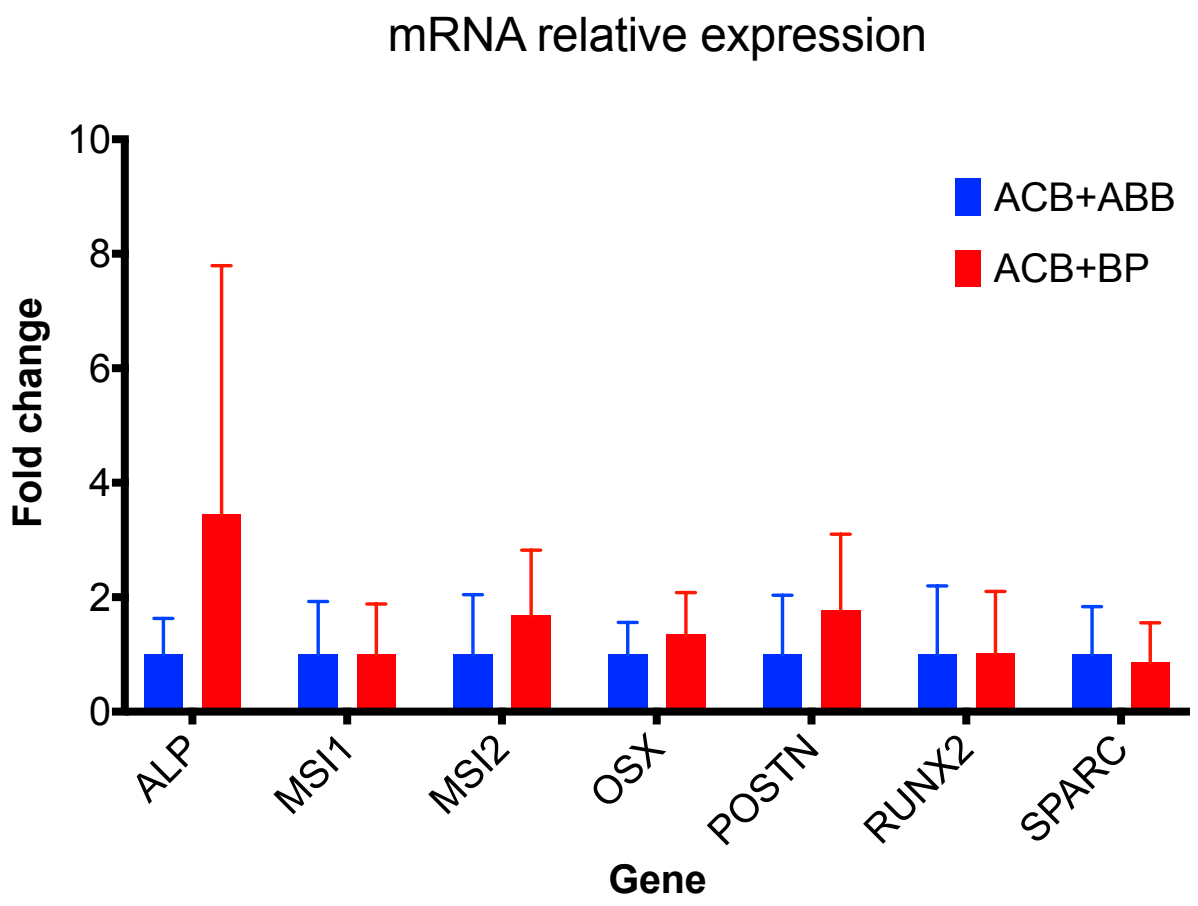

Figure 4: Panoramic views of biopsies from maxillary sinus grafted with A) ACB+ABB biomaterial, and B) ACB+BP biomaterial. Yellow dotted lines indicate the approximate interface between native and grafted bone. TB: Trabecular bone; nMT: non-mineralized tissue; MT: new mineralized tissue; ABB: anorganic bovine bone; BP: biphasic phycogenic biomaterial; Inf.In.: inflammatory infiltrate. Masson's Trichrome stain, bars: 500 micrometers.

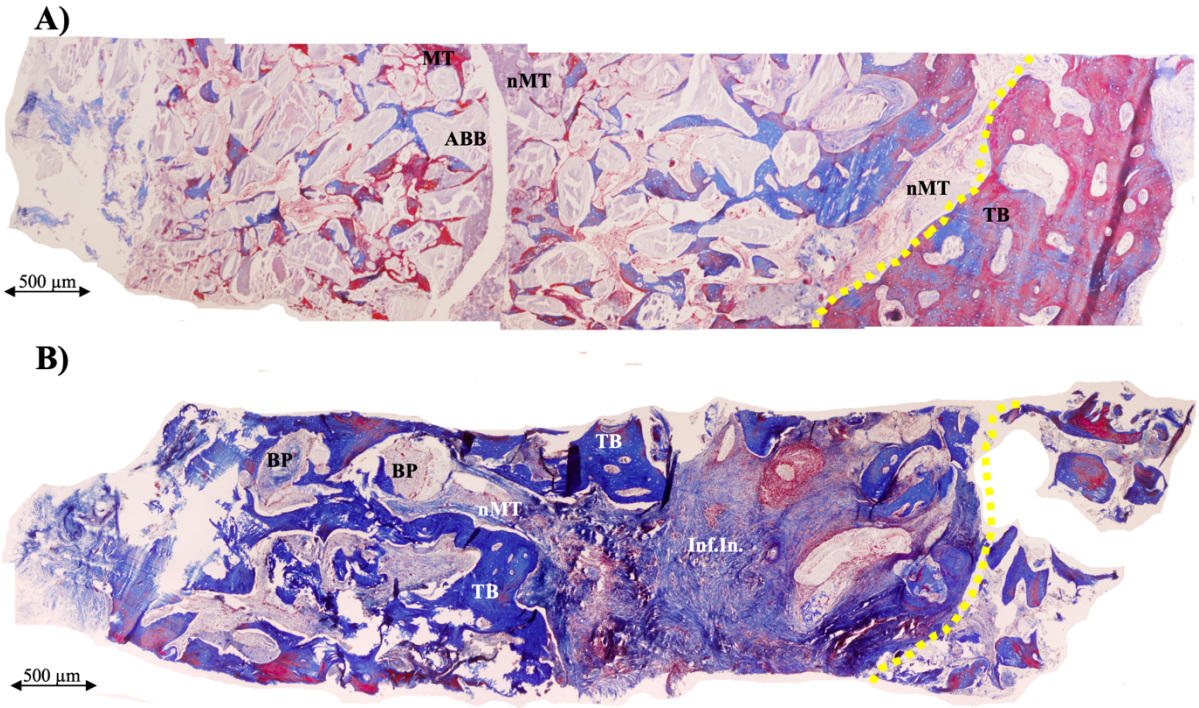

Figure 5: Detail of maxillary sinus graft biopsy. A) Remnant ABB biomaterial and trabecular bone (Masson's Trichrome stain, bars: 50 micrometers). B) Particle of biphasic phycogenic biomaterial next to the new bone formed and surrounded by abundant dense non-mineralized tissue. nMT: non-mineralized tissue; MT: new mineralized tissue; ABB: anorganic bovine bone; BP: biphasic phycogenic biomaterial. Masson's Trichrome stain, bars: 500 micrometers.

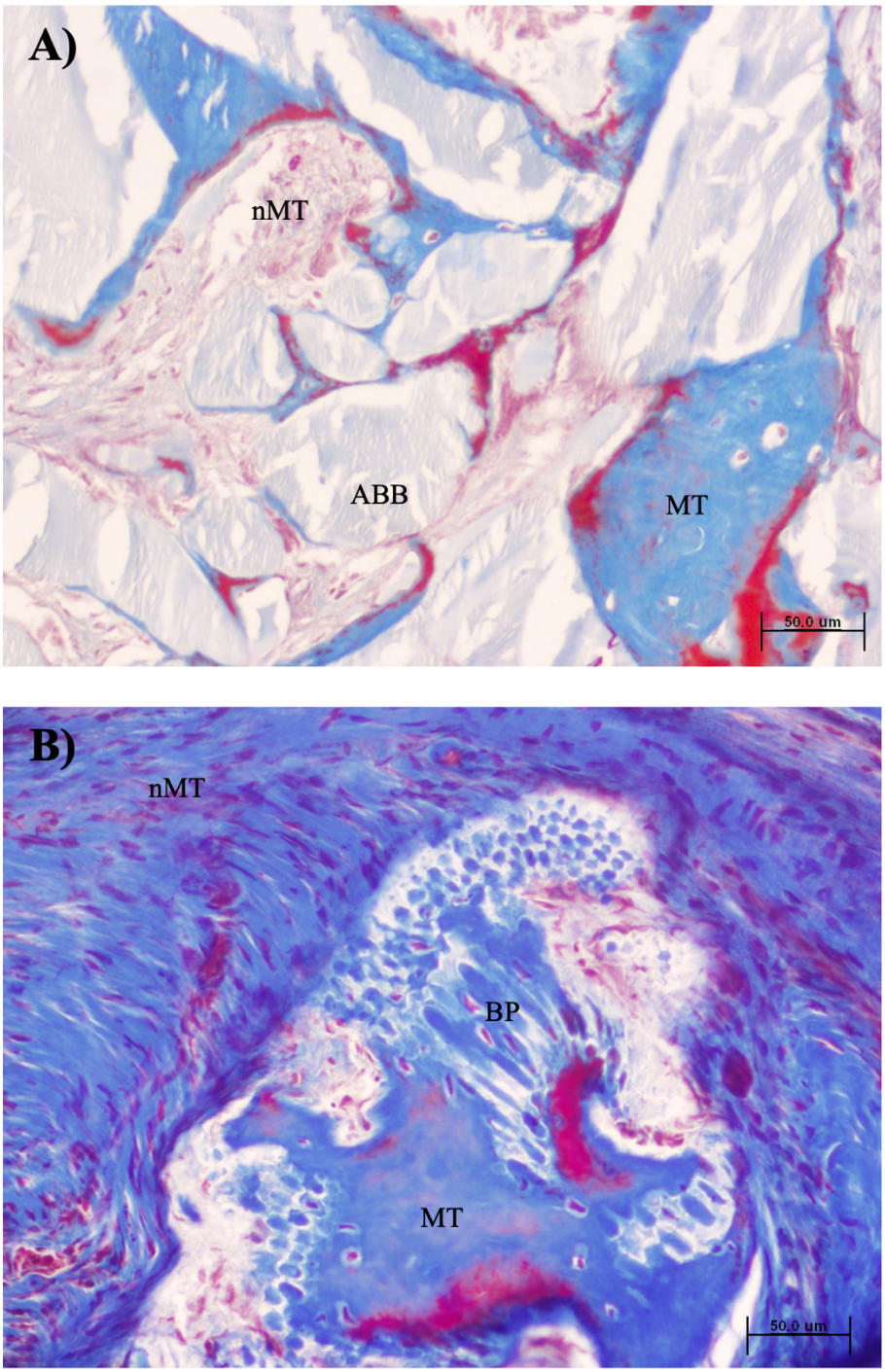

Figure 6: Immunohistochemical expression of Musashi-1 in A) ACB+ABB and B) ACB+BP case. Note the strong nuclear expression of MSI1 protein in the ACB+ABB group, and the absence in the ACB+BP. Bar: 50 micrometers (peroxidase-conjugated micropolymer and diaminobenzidine development).

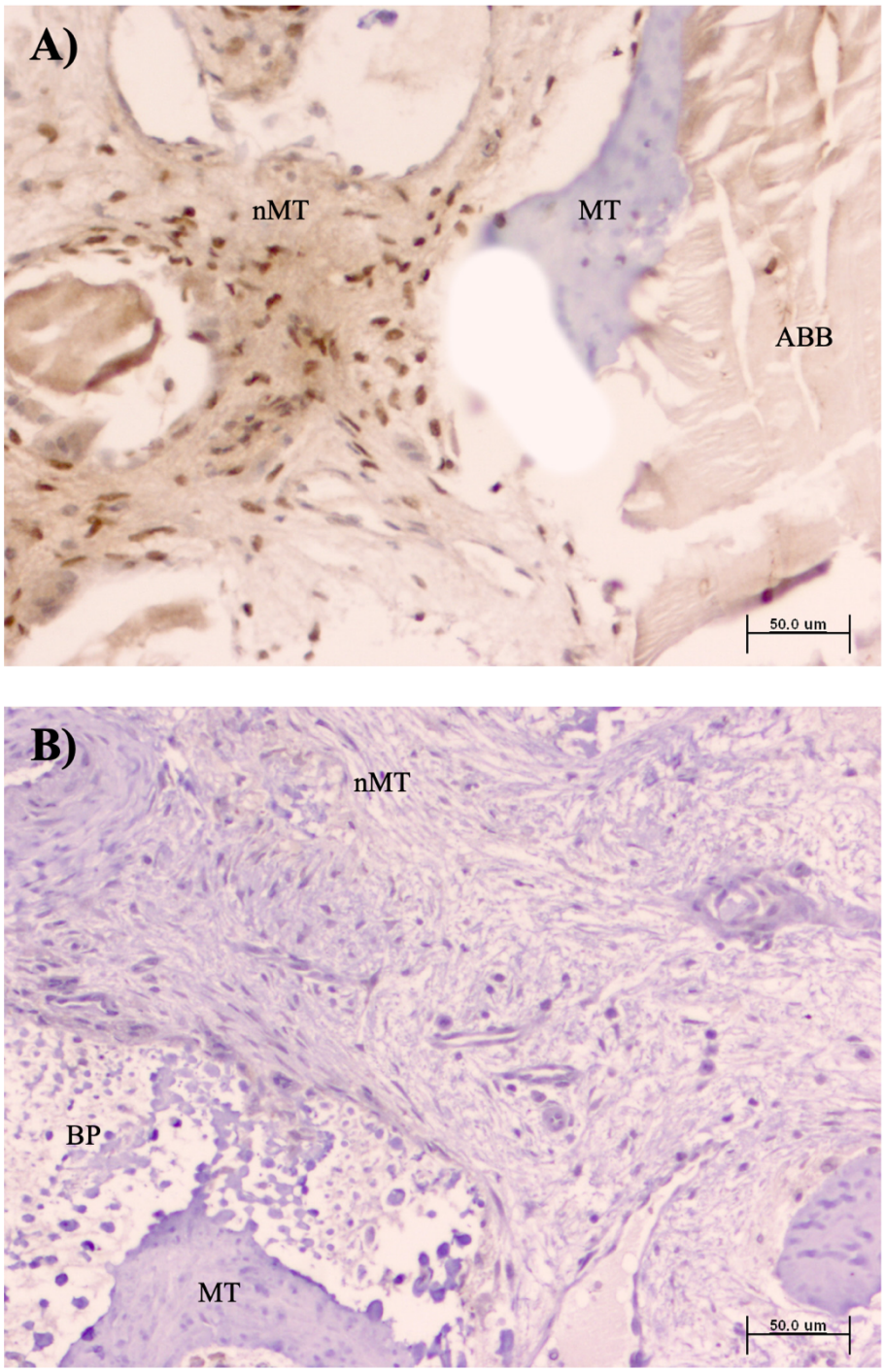

Figure 7: Semithin and ultrathin sections of maxillary sinus grafts biopsy. A) ABB particles are surrounded by a mix population of fibroblasts (F) and fibroblast-like (mesenchymal stromal cells, MSC) that do not show a developed rough endoplasmic reticulum nor collagen (C) in the vicinity. Osteoprogenitor cells are close to the biomaterial particles and newly formed mineralized tissue (MT) (A1 and A3) while fibroblasts are surrounded by collagen fibers (A2). B) BP particles are surrounded by a fibroblastic population (F) (B1 and B3), with evident organized extracellular matrix and fibrillar collagen (C) surrounding the particles and inside the pores of the biomaterial (B2).