HISTOPATHOLOGICAL COMPARISON OF HEALING AFTER MAXILLARY SINUS AUGMENTATION USING XENOGRAFT MIXED WITH AUTOGENOUS BONE VERSUS ALLOGRAFT MIXED WITH AUTOGENOUS BONE

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ABSTRACT

Objective: To compare the clinical and histologic outcomes of two different grafting materials (allograft and xenograft) when combined with autogenous bone and covered with a collagen membrane for sinus augmentation.

Material & Methods: A parallel case series of fourteen patients in need of a unilateral sinus augmentation was evaluated in this study. Seven patients received a graft composed by autologous cortical bone and anorganic bovine bone in a ratio of 1:1; the other seven patients received autologous cortical bone mixed with an allograft in the same ratio. Bone biopsies were obtained 6 months after sinus augmentation at the time of implant placement. Comparative histomorphometrical, histopathological and immunohistochemical analyses were conducted and statistically analyzed.

Results: After 12 months of functional loading, all implants in both groups were clinical and radiographically successful. Histomorphometrically, although the initial bone formation was not significantly different between groups (new mineralized tissue: 41.03(12.87)% vs. 34.50(13.18)%, p=0.620; allograft vs. xenograft groups), the graft resorbed faster in the allograft group (remnant graft particles: 9.83(7.77)% vs. 21.71(17.88)%; p=0.026; allograft vs. xenograft groups). Non-mineralized tissue did not statistically differ either (49.00(14.32)% vs. 43.79(19.90)%; p=0.710; allograft vs. xenograft groups). The histologic analyses revealed higher cellular content, four times more osteoid lines and higher vascularization in the xenograft group. Musashi-1 (mesenchymal stromal cell marker) was also more intensively expressed in the xenograft group (p=0.019).

Conclusions: Both composite grafts generate adequate substratum to receive dental implants after healing. Compared with the xenograft composite, allograft composite shows faster turnover and a quicker decrease in biological action after 6 months.

INTRODUCTION

Biomaterial usage is a common practice by dental professionals for promoting bone regeneration. Current knowledge advocates for the use of biomaterials in almost all of our surgical procedures: from a simple tooth extraction to the most complex oral rehabilitation. Thus, it is imperative to clarify the biological behavior of such biomaterials and combinations and not just expose the clinical outcomes. Maxillary sinus augmentation procedures represent an excellent model for studying graft behavior in humans. A biopsy can be taken at the moment of implant installation with no ethical contraindication.

Xenografts, bone substitutes of animal origin (such as Bio-Oss®, Geistlich Pharma, Wolhusen, Switzerland; anorganic bovine bone, ABB), have been widely studied alone or in combination with autologous bone for successful maxillary sinus floor elevation (Hallman et al. 2002; Jensen et al. 2012). This biomaterial has demonstrated excellent outcomes in terms of space maintaining and induction of bone formation. However, ABB particles undergo very slow resorption rates (Galindo-Moreno et al. 2013). Thus, the amount of new mineralized tissue could be lower compared to other bone substitutes (Orsini et al. 2005; Froum et al. 2006; Jensen et al. 2012). To overcome this potential limitation, other biomaterials with faster resorption rates have been proposed. Bone allografts can be produced by solvent dehydration (SDBA) (Puros Allograft®, Zimmer Dental Inc., Carlsbad, CA, USA) or freeze-dried (FDBA) (MinerOss®, Biohorizons, Birmingham, AL, USA). Manufacturing procedures are different for both bone substitutes, so it is plausible to expect them to have different properties (Monje et al. 2017). While for SDBA there are several studies comparing its capacity to augment the posterior atrophic maxilla, FDBA presents limited and variable information ranging from 31.8% of new mineralized tissue and 8.49% residual graft at 6 months (Gapski et al. 2008) to 23.02% of new mineralized tissue and 22.25% of remaining allograft (Avila et al. 2010a).

But not only tissue compartments should be studied. Cellularity is a very important parameter that demonstrates the pathway of the graft remodeling. Musashi-1 (Msi1) is a 39 kDa and 362 amino acids RNA-binding protein with two ribonucleoprotein motives (RBD1 y RBD2). It can be used as an adult stem cell marker (Nagata et al.

1999). Msi1 regulates the maintenance and differentiation of stem/precursor cells through the post-transcriptional regulation of its target RNA (Kaneko et al. 2000; Okano et al. 2002). It also regulates osteogenic differentiation in vitro through the Wnt1 pathway (Hong & Kang 2013). In fact, it seems to also be related to the activity of Runx2, a nuclear gene regulatory effector that supports the transduction of extracellular osteogenic signals into the nucleus (Galindo et al. 2005).

The aim of the present study was to evaluate histopathological, histomorphometrical, and immunohistochemical differences in bone healing in human maxillary sinus floor elevation procedures using a combination of xenograft and autologous bone versus a combination of allograft and autologous bone.

MATERIALS & METHODS

Study design

The study was designed according to the STROBE guidelines for observational studies as a one center prospective two parallel arms case series study with specific inclusion and exclusion criteria. This study was reviewed and approved by the Ethics Committee for Human Research of the University of Granada (approval date: March 10th, 2009). All patients received detailed oral and written information on the study and signed a written consent before any study activity was initiated.

Patients admited at a Dental Faculty practice of the University of Granada were recruited for the study after signing their informed consent. Subjects for the study were selected according to the following inclusion criteria: patients systemically healthy who did not take any drugs at least two weeks before the surgery, edentulous in the maxillary posterior sector and with less than 5 mm of remaining alveolar bone height. Periodontal patients (four or more teeth with one or more sites with PD ≥4 mm and CA loss ≥3 mm) or patients suffering from any disease known to alter bone metabolism as well as pregnant women were excluded from the study.

Surgical and restorative procedure

All patients were prescribed with 875/125 milligrams of amoxicillin/clavulanic acid every 8 hours starting one day prior to the surgery and for 7 days post-surgery. Lateral window access technique modified by Galindo-Moreno and co-workers was

used to perform all the surgeries (Galindo-Moreno et al. 2007). Briefly, a curve bone scraper (Safescraper®, Meta, Reggio Emilia, Italy) with 2.5 cc of capacity was used to collect maxillary autologous cortical bone (ACB) and to expose the Schneiderian membrane in all the procedures. Seven patients were grafted with a mix of ACB and xenograft, specifically anorganic bovine bone (ABB; 250 to 1000 μm particle size; Bio-Oss® - Geistlich Pharma AG, Wolhusen, Switzerland) in a 1:1 ratio (ACB+ABB group). Another group of seven patients received a mixture of ACB and a combination of cortical and cancellous allograft particles (FDBA) (MinerOss® - Birmingham, AL, USA) in the same 1:1 ratio (ACB+FDBA group). After the bone graft was in place, an absorbable collagen membrane (Bio-Gide® - Geistlich Pharma AG) was placed over the lateral aspect of the bony window and the flap was carefully repositioned with 3/0 surgical silk (Laboratorio Aragó, Barcelona, Spain). In all cases primary wound closure was achieved.

After a 6-month healing period, a 3 mm internal diameter trephine was used to collect bone biopsies for histological analysis in the same surgical location where implants (OsseoSpeed® TX, Dentsply Sirona Implants, Mölndal, Sweden) were later placed.

Clinical variables

Data was collected on age, gender, alcohol consumption (non-drinker or >10 g of ethyl alcohol daily), and smoking habits (0 cigarettes/day: non-smoker; 1-10 cigarettes/day: mild smoker; >10 cigarettes/day: heavy smoker).

Histopathological study

The trephine biopsies were immersed in a 10% buffered formalin solution for 24 h, decalcified in a formaldehyde (10% w/v), formic acid (8% w/v) and methanol (1% w/v) solution (Decalcifier I®, Surgipath® Europe Ltd., Peterborough, UK) for 24 h at 37ºC in an oven and then embedded in paraffin. 4-μm sections were cut along the central axis of the biopsies, dewaxed, rehydrated and stained with hematoxylin-eosin, Masson trichrome and Peryodic Acid Schiff. A millimeter scale in the eyepiece of a BH2 microscope (Olympus Optical Company, Ltd., Tokyo, Japan) with a 40x objective was used to count vessels, mesenchymal stromal cells, osteoblast, osteoclast and osteocyte cells per mm^2.

Masson trichrome-stained sections were used for semi-automatic bone histomorphometry applying ImageJ® software (NIH, USA, http://rsb.info.nih.gov/ij/) to 10 randomized images captured with a 10x objective in a microscope with a DP70 digital camera (Olympus). Separate quantifications of areas (in mm^2 and percentages of total area) of mineralized tissue, remnant particles of ABB and non-mineralized tissue were done.

Immunohistochemical analysis

Sections were dewaxed, rehydrated, and heat-treated for antigenic unmasking in a 1 mM EDTA buffer, pH=8 in a PT module (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 95ºC for 20 min. Sections were then incubated for 30 min at room temperature with pre-diluted CD34 monoclonal antibody (clone QBEND/10) to identify endothelial cells, CD44 monoclonal antibody (clone 156-3C11) to identify osteocytes and inflammatory cells, vimentin monoclonal antibody (clone V9) to identify mesenchymal cells (positive control), and tartrate-resistant acid phosphatase (TRAP) (clone 26E5) to identify osteoclasts cells. Other sections were incubated for 1h at room temperature with the prediluted polyclonal antibodies against Runx2 (1:80 dilution; SantaCruz Biotechnology Inc., CA, USA) and Musashi-1 (1:100 dilution; Sigma-Aldrich, Barcelona, Spain). All antibodies were purchased from Master Diagnóstica (Granada, Spain). The micropolymer-peroxidase-based method (Ultravision Quanto, Thermo Fisher Scientific Inc.) was applied for the immunohistochemistry study with an automatic immunostainer (Autostainer 480, Thermo Fisher Scientific Inc.), followed by development with diaminobenzidine (Master Diagnóstica).

Results of the immunohistochemical analysis were calculated in a semiquantitative manner by using a scale of 0 to 3 (0=absence; 1=mild [<10% positive cells]; 2=moderate [10 to 25%]; 3=intense [> 25%]). For each sample, the expression in the sample as a whole and separately for fibroblast-like mesenchymal stromal cells, endothelial cells and adipocytes was evaluated. Also, a millimeter scale in the eyepiece of a BH2 microscope (Olympus) with a 40x objective was used to count the number of positive cells/vessels per mm^2.

Statistical analysis

A specific software program IBM SPSS-Windows (v. 20.0; IBM SPSS, Armonk, NY, USA) was used for the analyses. After descriptive analysis, the Mann-Whitney U-test or Chi-square test were used to evaluate the significance of differences, considering $p<0.05$ to be significant.

RESULTS

A total of fourteen unilateral maxillary sinuses were grafted. All grafts and implants in both groups integrated successfully, and were functional after a 12-month loading period according the criteria from the Pisa Consensus Conference (Misch et al. 2008), i.e., the implants showed no pain or tenderness upon function, no mobility, less than 2 mm of radiographic bone loss from initial surgery and no history of exudates. None of the patients suffered sinus membrane perforation during the surgery or developed maxillary sinusitis. **Table 1** shows demographic variables.

Morphological features

One end of all the biopsies showed the presence of compact, mature cortical bone, which could be easily differentiated from the grafted area. In every specimen of ACB+ABB (7/7 cases, 100%), it was constant to observe almost every particle of residual graft surrounded by newly formed mineralized tissue with osteoblasts lining it (**Figure 1A**). No resorption phenomena was detectable in pristine bone. However, although the ABB particles seemed to undergo a very slow resorption process (**Figure 2A**), a high number of multinucleated osteoclasts-like cells (TRAP positive) surrounded the particles (**Figure 3A**). Signs of tissue inflammation and lymphocytes/macrophages cells were not observed. New non-mineralized tissue was formed by numerous fibroblasts-like cells and vessels.

ACB+FDBA showed a similar newly formed mineralized tissue with scarce residual graft particles and a lower number of vessels and fibroblast-like cells. FDBA particles seemed to undergo a very fast resorption process. Remaining particles were present in 50% of the images (36/70 of fields, magnification 10x) (**Figure 1B, 2B**) with few multinucleated TRAP-positve cells (**Figure 3B**).

Histomorphometrically, new mineralized tissue formation was quicker in the ACB+FDBA group but not statistically significant (41.03(12.87)% vs. 34.50(13.18)%; p=0.620, Mann Whitney U-test; ACB+FDBA vs. ACB+ABB) (**Table 2**). A more intense resorption of the biomaterial was also observed in the ACB+FDBA group (9.83(7.77)% vs. 21.71(17.88)%; p=0.026, Mann Whitney U-test; ACB+FDBA vs. ACB+ABB) (**Figure 2**). Non-mineralized tissue did not statistically differ either (49.00(14.32)% vs. 43.79(19.90)%; p=0.710, Mann Whitney U-test; ACB+FDBA vs. ACB+ABB). The histologic analysis revealed a significantly higher cellular presence, four times more osteoid lines and higher vascularization in the ACB+ABB group (**Table 3**) (**Figure 2**).

In maxillary sinus augmentation with ACB+ABB the Msi1 expression in MSCs was moderate/intense (2.2(0.83)) (**Figure 4A**), whereas it was mild or absent (0.8(0.75)) in the ACB+FDBA group (p=0.019, Mann Whitney U-test) (**Figure 4B**). No differences were observed in the other cell types (**Table 4**). Similarly, the expression of Runx2 was higher in cases grafted with ACB+ABB (**Figure 5A**) but with no statistically significant difference (1.42(1.13) vs. 0.83(0.75); p=0.298, Mann Whitney U-test) (**Table 4**) (**Figure 5B**).

DISCUSSION

New mineralized tissue formed after bone grafting has been regarded as "*new vital bone*". Thus, grafts that induce a higher quantity of the mineralized component have been associated with higher quality. However, vital is everything in the bone, not just the mineralized portion. In fact, the analysis of the non-mineralized tissue compartment is as important, and potentially even more, than the other tissue compartments. This non-mineralized tissue is described in the literature as "*soft connective tissue*". Understanding the bone marrow or non-mineralized tissue compartment is essential to understanding bone homeostasis, reparation and maturation processes. Cellular and vascular components are present in this portion of the bone. For this reason, in the current manuscript we have focused on analyzing the composition of this area in two different composite grafts.

The biomaterial strongly influences the host response by regulating specific biological properties. In fact, recent systematic reviews highlight the differences in terms of

different tissue compartments formed after sinus floor elevation with different biomaterials and time points (Danesh-Sani et al. 2016; Corbella et al. 2016). Moreover, other reviews also made similar observations although it was reported that after 9 months, the differences are not evident anymore (Handschel et al. 2009; Lundgren et al. 2017). Thus, it can be confirmed that both timing and biomaterial are key factors in the healing after sinus floor elevation. As a matter of fact, although some protocols and new materials are currently advocating for shorter healing times, these are still not the standard of care. In the particular case of the combination of autogenous bone and xenograft, healing times shorter than 6 months have shown less favorable outcomes than 6 months or more in terms of new bone formation (Danesh-Sani et al. 2016; Corbella et al. 2016).

This study evaluates histological and histomorphometrical differences between two graft composites. Our results show that after 6 months of healing the combination of allogeneic plus autologous bone in a 1:1 ratio promotes 41.03(12.87)% of mineralized tissue, 49.00(14.32)% of non-mineralized tissue, and 9.83(7.77)% of residual graft. On the other hand, the combination of xenogeneic plus autologous bone in the same proportion promotes less mineralized and non-mineralized tissue (34.50(13.18)% and 43.79(19.90)%, respectively) but maintains higher residual graft particles (21.71(17.88)%). Previous studies have found no differences in terms of the clinical and histomorphometric outcomes when using ACB+ABB (Mordenfeld et al. 2014) or ACB+FDBA (Beitlitum et al. 2010) for alveolar ridge reconstruction, in contrast with the findings of the current study. Other studies on sinus augmentation with the use of FDBA alone show a lower percentage of mineralized tissue, ranging from 23.02% (Avila et al. 2010a) to 31.8% (Gapski et al. 2008). Similar findings have been reported with the use of ABB or ACB+ABB. Previous reports found a lower proportion of mineralized tissue with lower proportion of autogenous bone in the graft (Hallman et al. 2002; Thorwarth et al. 2006). In fact, during the analysis of different proportions of ACB in the composite graft (50:50 vs. 80:20), our previous results confirm better outcomes when more autogenous bone was used (Galindo-Moreno et al. 2011). Although there are reports indicating no differences with the addition of ACB to the mix (Schmitt et al. 2015; Alayan et al. 2016), we have found the opposite. The differences might be due to the addition in our series of ACB, which could

improve the early graft consolidation. It has been suggested that the vitality of autografts is not evident because the majority of the osteocytes do not survive the grafting procedure (Zerbo et al. 2003). However, many other factors transplanted with the autogenous graft could play an important role in the early healing, such as endogenous BMPs and necrosis factors that could induce tissue reparation (Roberts & Rosenbaum 2012). All of our samples in the ACB+ABB group were surrounded by new mineralized tissue. When used without autogenous bone, ABB is surrounded by new mineralized tissue in significantly lower proportion (42.0±26.8 vs. 19.6±27.3) (Alayan et al. 2016).

Allografts have been studied in the past in comparison to xenografts. A prospective, blinded, randomized study on 13 subjects reported better outcomes with the use of allograft (Puros®) compared to anorganic bovine bone (Bio-Oss®) (Froum et al. 2006). The reported bone volume for the allograft was 35.90% (28.25% of new mineralized tissue + 7.65% of remaining graft particles) and 12.44% for the xenograft. In the case of the xenograft, the authors, arguably, did not account for the amount of remaining particles, as in the case of the allograft, justifying that the anorganic bovine particles should not be considered as bone. If accounted for, the bone volume would have been 45.44%, higher than with the allograft. This tendency was confirmed with similar results in a microradiography study. Although the mineralized component was 46.26±8.11% for ABB and 35.41±2.81 for allograft, the new mineralized tissue volume, excluding the remaining particles, was 24.90±5.67% for the ABB group and 35.41±2.78% for the allograft group (Schmitt et al. 2013). Again, it seems that separate quantification between new mineralized tissue and remaining graft particles have been done only for the ABB group. This is possibly due to microradiographs not being suitable for distinguishing between allograft particles and new mineralized tissue. Furthermore, microradiography is not sensitive enough to distinguish between the different maturation stages that the tissues might be at. In this sense, Lee and co-workers reported that ABB (Bio-Oss®) produces a more rigid bony structure than the allograft (Puros®) (Lee et al. 2009). In fact, a large scale study comparing 13 different biomaterials in 295 patients subjected to maxillary sinus floor elevation reported that anorganic bovine bone generates the greatest amount of new mineralized tissue and lowest marrow spaces (Traini et al. 2015).

But new mineralized tissue formation is not the only important aspect. In the current study, only the biomaterial resorption showed statistically significant differences. As expected, in spite of being a combination of mineralized cortical and cancellous bone, the allograft showed a higher resorption rate than the ABB (9.83% vs. 31.84% of remaining particles, respectively). Interestingly, it has been widely reported that Bio-Oss® undergoes a very slow resorption mediated by multinucleated osteoclasts-like cells that are TRAP and cathepsin k positive (Galindo-Moreno et al. 2013). Ideally, every biomaterial should be resorbed according to the classic definition (Boyne 1973). However, the persistence of the biomaterial could be considered to improve the new bone features if it is integrated and functional in the environment. For example, by getting re-vascularized (Galindo-Moreno et al. 2010) and recolonized with the host's cells (Galindo-Moreno et al. 2014). These findings highlight once more that every material should have different clinical applications depending on the expected effect that the clinician demands. A biomaterial might not always be used as a "gold standard" in all the clinical situations.

Globally, our histomorphometric outcomes for both groups are in the range of different studies compiled recenlty by Corbella and coworkers (Corbella et al. 2016) and very similar to the average values calculated in a different meta-analysis (Danesh-Sani et al. 2016). The main problem with these studies is that the histomorphometric analysis only shows the proportion of the different bone compartments. However, what happens in each compartment, and particularly in the non-mineralized tissue, is of paramount importance. Most vessels and cells are in this portion of the tissue. Thus, studying them is key.

Vascularization, as it is known, is very important for the grafted area to repair (Padial-Molina et al. 2015). Moreover, vascularization and mineral deposition are considered a paired processes (Parfitt 2000). That is why some therapies work towards improving this aspect together with the mineral deposition and graft remodeling (Huang et al. 2005; Kaigler et al. 2015). The osteoconduction promoted by each biomaterial depends on the number and distribution of the new blood vessels in the non-mineralized tissue (Davies 2003). In this sense, each biomaterial induces different levels of tissue neovascularization (Boeck-Neto et al. 2009). In the current study, the ACB+ABB group presented with almost double the blood vessels

(86.63(42.44) vessels/mm^2) than the allograft composite (52.06(15.60) vessels/mm^2). The results concerning the ACB+ABB group are similar to others reported previously by our group (Galindo-Moreno et al. 2010) and much higher than others using ABB or ACB alone (Degidi et al. 2007). Regarding allografts, the neovascularization in the current study is also higher than those reported previously (Boeck-Neto et al. 2009), surely related to the biologic action of the autogenous bone added to our composite. This increased vascularization in the ACB+ABB compared to the ACB+FDBA group could explain the differences in the new osteoid lines, which are four times higher in the first combination (21.20(11.60) vs. 5.14(5.52), respectively). This is directly related to a higher osteoconductive and reparative potential that can be maintained for a longer time.

The analysis of the histological and immunohistochemical characteristics demonstrates the vital structure and potential future events in the grafted area. In this sense, beside the higher number of osteoid lines and vessels previously mentioned, a higher cellularity per mm^2 was also observed in the non-mineralized tissue of the ACB+ABB composite. This suggests higher activity and tissue remodeling. Interestingly, in spite of presenting a smaller proportion of non-mineralized tissue in the xenogeneic composite, the number of mesenchymal stromal fibroblast-like cells per mm^2 almost duplicates that in the allogeneic composite, with statistical significance (291.70(64.18) vs. 169.11(142.62) cells/mm^2, respectively; p=0.038). Previous studies from our group and others confirm these observations (Galindo-Moreno et al. 2007; Degidi et al. 2007; Galindo-Moreno et al. 2010). As also mentioned above, the use of ACB is particularly important as it induces higher cellularity (Hallman et al. 2002; Thorwarth et al. 2006; Galindo-Moreno et al. 2011). The biological relevance of these cell types is still needs further investigation. To explore this aspect we analyzed the expression of Musashi-1 and Runx2. We found higher Msi1 expression in the mesenchymal stromal fibroblast-like cells in the ACB+ABB group, which may indicate a more osteogenic differentiating environment, although the expression of Runx2 was not statistically different.

Musashi-1 is an adult stem cell marker that binds to RNA (Nagata et al. 1999) to maintain the stem cell division potential (Okano et al. 2005) by reducing Notch-1 expression (Sureban et al. 2008). Its specific role in bone regeneration is still mostly

unknown but it seems to be related to the induction of Runx2, a potent inductor of osteoblast differentiation to mature functional cells (Zerbo et al. 2005) over surfaces with diverse proteins adsorbed, such as osteopontin (Galindo-Moreno et al. 2015). In the current study, the higher cellularity, number of blood vessels and osteoid lines in the ABB group was also associated with higher detection of Msi1 and Runx2 in cells in that group compared to the other. This may indicate that these two markers are in fact associated with the regeneration process and that the combination of xeno and autograft actually increases these activities.

This study has some limitations that are important to keep in mind when interpreting our data. The number of patients is limited, although compared with other published manuscripts, this study is in the same range. This is important in order to balance different patient's characteristics but also from the site specific configuration. Anatomic variables of the maxillary sinus are numerous and associated with other clinical parameters (Velasco-Torres et al. 2016, 2017). These anatomical variations have been related to the response to the graft at the histomorphometrical level (Avila et al. 2010b). At the end, the further from the bony walls, the slower the maturation of the graft (Busenlechner et al. 2009). However, we have not particularly analyzed these variables in the current study. In addition, a split-mouth design could improve the outcomes of the comparison considering that several patient-related factors such as smoking/drinking habits, type of edentulism or history of periodontitis influence the response to the graft in maxillary sinus floor elevation (Galindo-Moreno et al. 2012).

CONCLUSIONS

In conclusion, both composite grafts generate an adequate substratum to receive dental implants after healing. Allograft and autograft composite shows a quicker turnover, although its biological activity demonstrated by the cellularity is lower after 6 months in comparison with the xenograft and autograft composite.

COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of Interest

The authors declare no conflict of interest, either directly or indirectly, in any of the products listed in the manuscript.

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Ethical aspects

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

This line of research was reviewed and approved by the Ethics Committee for Human Research of the University of Granada (approval date: March 10th, 2009).

All patients received detailed oral and written information on the study and signed a written consent before any study activity was initiated.

TABLES

Table 1: Demographic and clinical variables.

	ACB+ABB	ACB+FDBA	p-values
Age	64.14(45-78)	62.71(54-78)	0.799*
Gender (% M/F)	57.14/42.86	42.86/57.14	0.500**
Smokers (% >10 cigarettes/day)	28.57	57.14	0.296**
Periodontal disease free (%)	100	100	-

Values are expressed as mean(standard deviation) or percentage; *Mann-Whitney U-test; **Chi-square test.

Table 2: Morphometric comparison study in graft component between groups.

	ACB+ABB		ACB+FDBA		p-values*
	mm^2	%	mm^2	%	
Mineralized tissue	0.178(0.06)	34.50(13.18)	0.210(0.06)	41.03(12.87)	0.620
Residual graft	0.112(0.07)	21.71(17.88)	0.044(0.02)	9.83(7.77)	0.026
Non-mineralized tissue	0.226(0.06)	43.79(19.90)	0.245(0.08)	49.00(14.32)	0.710

Values are expressed as mean(standard deviation); *Mann-Whitney U-test.

Table 3: Immunohistochemical comparison study of microscopic component between groups.

	ACB+ABB	ACB+FDBA	p-values*
Osteoblasts/CD56+(mm²)	244.24(233.47)	36.38(41.15)	0.053
Osteoclasts/TRAP+ (mm²)	184.33(111.91)	2.99(3.14)	0.043
Osteocytes/CD44+ (mm²)	926.26(735.59)	139.38(32.64)	0.001
Osteoid lines (no./biopsy)	21.20(11.60)	5.14(5.52)	0.018
Vessels/CD34+ (mm²)	86.63(42.44)	52.06(15.60)	0.165
MSCs/Musashi1+ (mm²)	291.70(64.18)	169.11(142.62)	0.038

Values are expressed as mean(standard deviation); *Mann-Whitney U-test.

Table 4: Comparative immunohistochemical expression of Msi1 and Runx2 in different cells of two type of maxillary sinus grafts.

	Msi1			Runx2		
	ACB+ABB	ACB+FDBA	p-values*	ACB+ABB	ACB+FDBA	p-values*
Osteoblasts	0.8(0.83)	0.66(0.81)	0.796	1.0(1.0)	0.33(0.51)	0.170
Osteoclasts	0.0(0.0)	0.0(0.0)	-	0.0(0.0)	0.0(0.0)	-
Osteocytes	0.8(0.33)	0.83(0.81)	0.375	0.0(0.0)	0.0(0.0)	-
MSCs	2.2(0.83)	0.8(0.75)	0.019	1.42(1.13)	0.83(0.75)	0.298

Values are expressed as mean(standard deviation); *Mann-Whitney U-test.

FIGURE LEGENDS

Figure 1. Panoramic trephine core biopsies A) Anorganic bovine bone with autograft and B) freeze-dried bone allograft with autograft (Masson trichrome stain x 0.2).

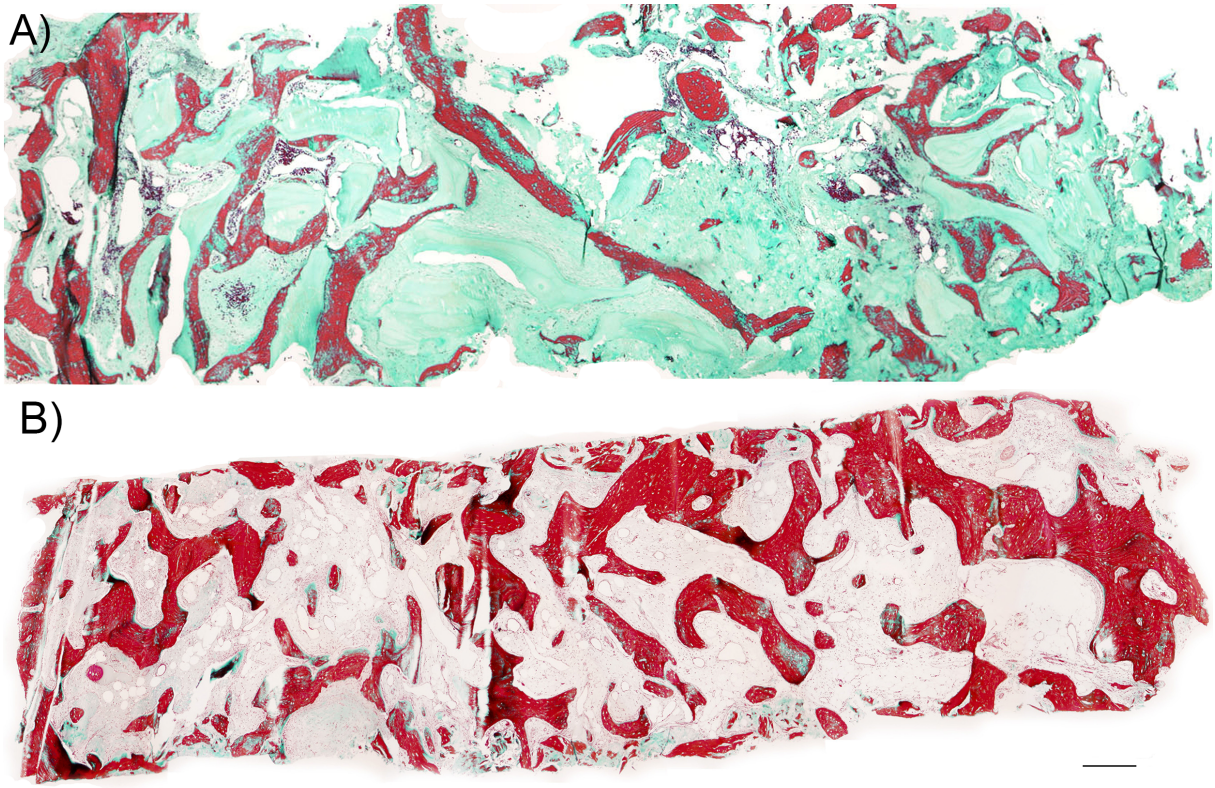


Figure 2. Different components of bone graft. NewMT: New mineralized tissue, P: particles of biomaterial, NonMT: non-mineralized tissue. A) Anorganic bovine bone with autograft and B) freeze-dried bone allograft with autograft (Masson Trichrome stain x 4).

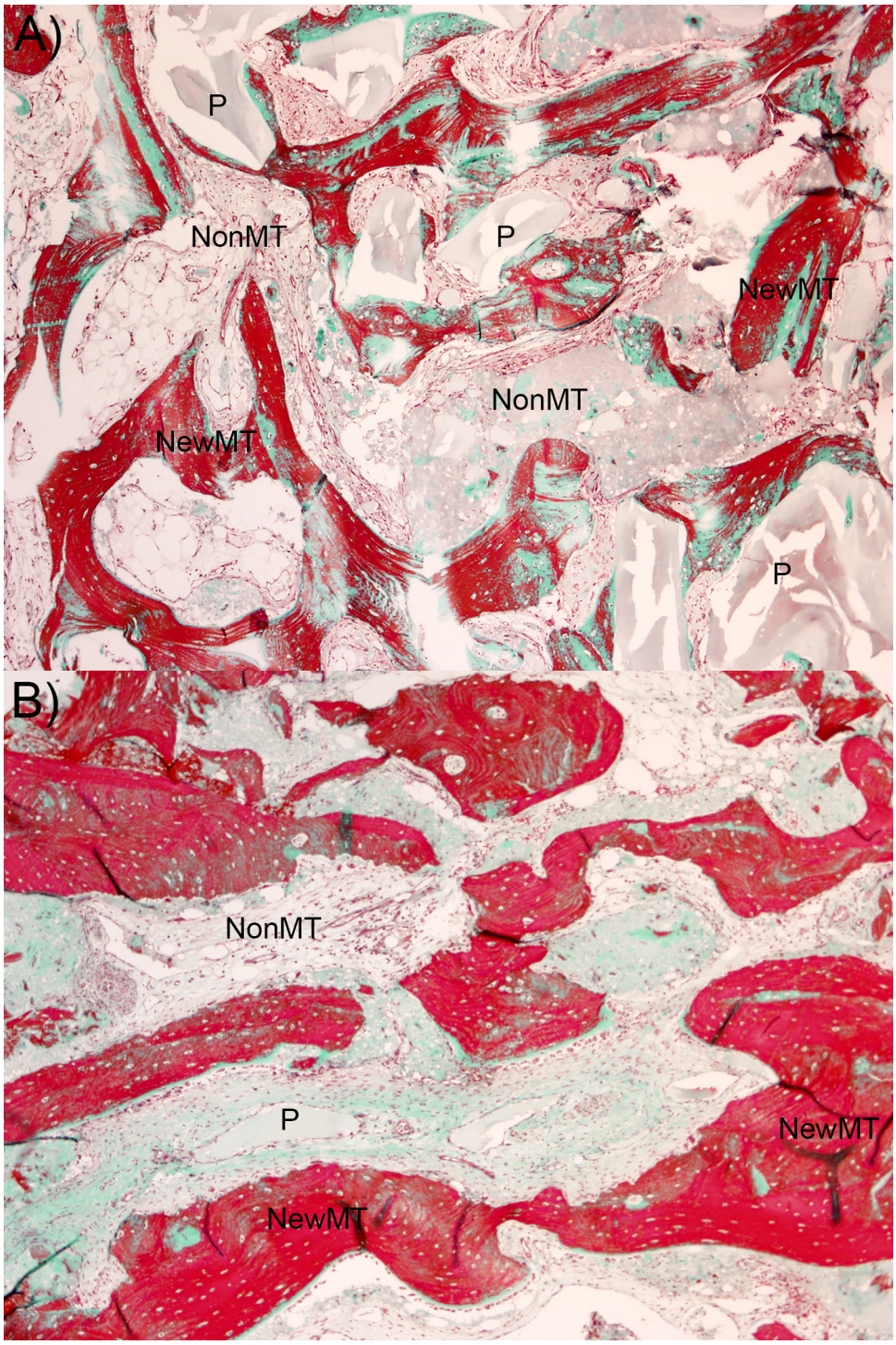


Figure 3. Immunohistochemical expression of TRAP. A) Anorganic bovine bone with autograft (micropolymer peroxidase-conjugated, original magnification x10) and B) freeze-dried bone allograft with autograft (micropolymer peroxidase-conjugated, original magnification x4).

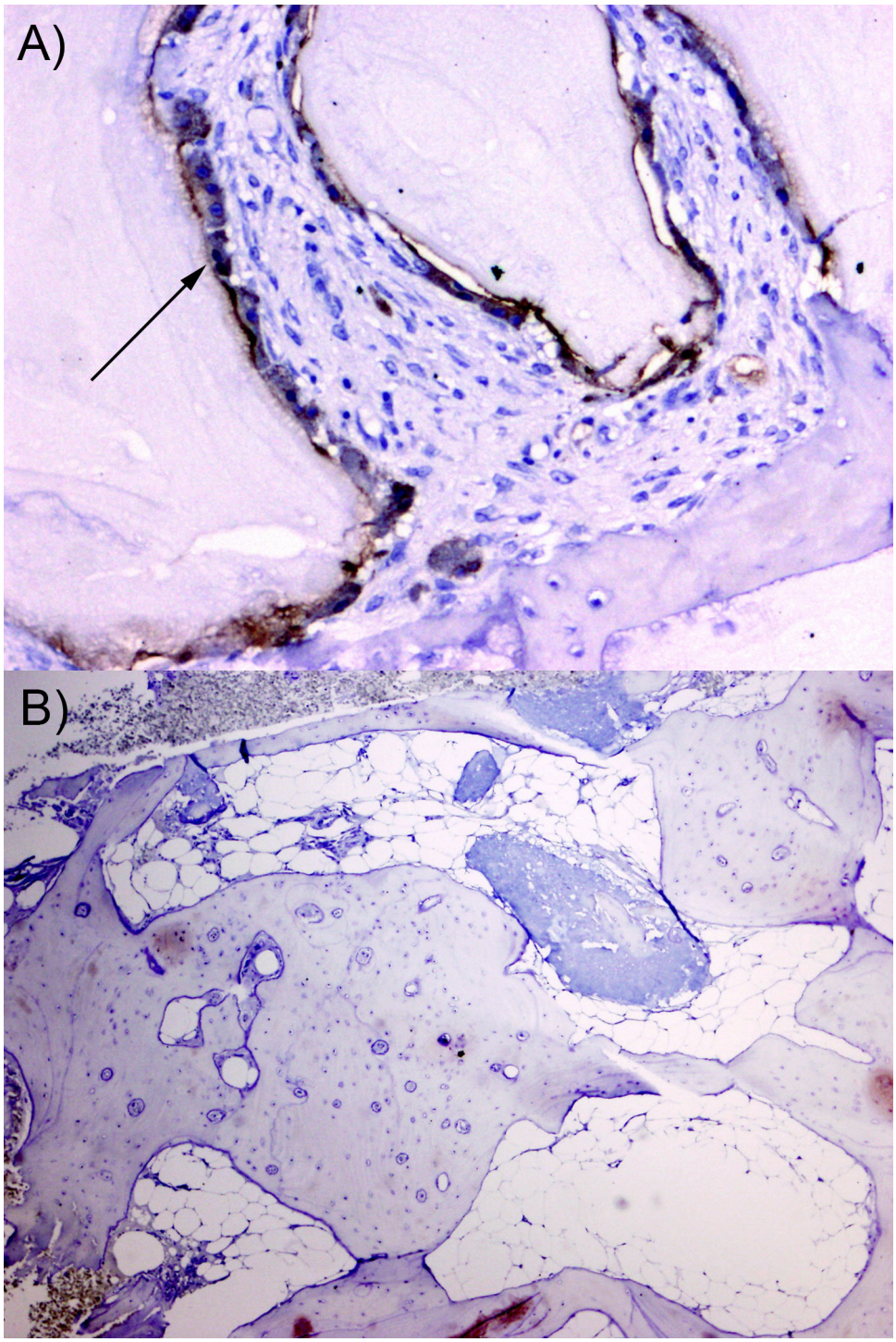


Figure 4. Immunohistochemical expression of Msi1. A) Anorganic bovine bone with autograft (micropolymer peroxidase-conjugated, original magnification x20) and B) freeze-dried bone allograft with autograft (micropolymer peroxidase-conjugated, original magnification x10).

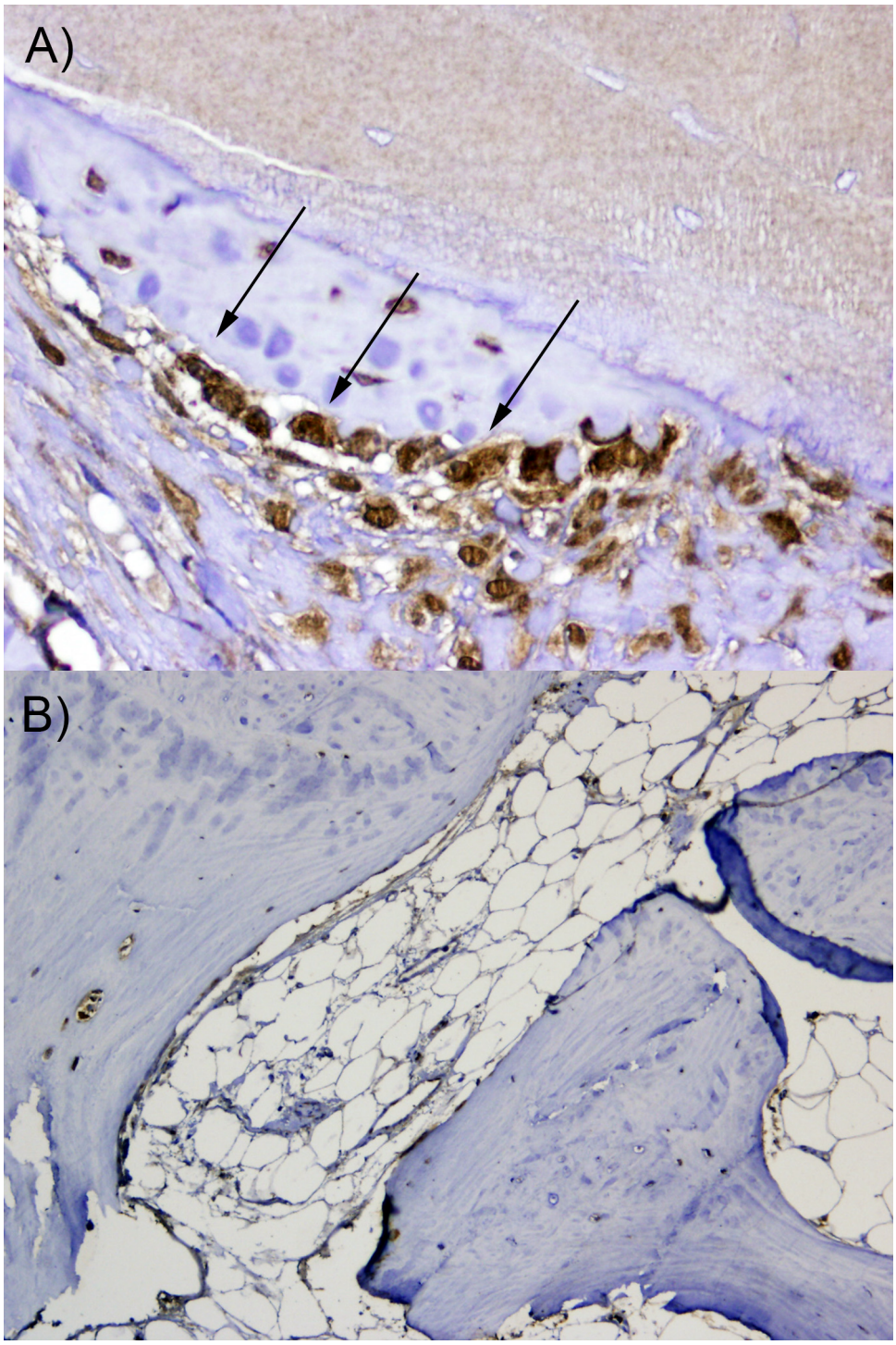
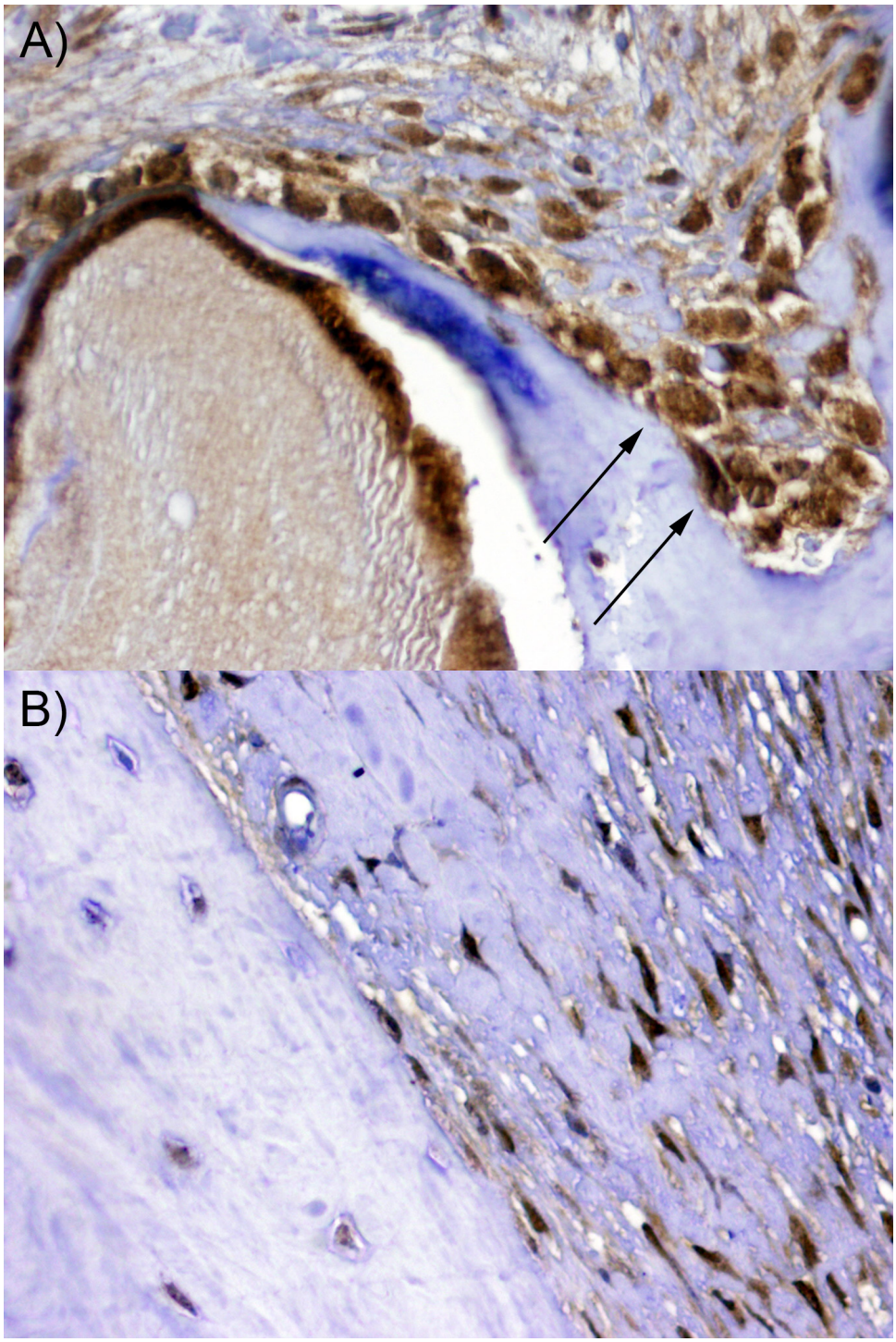


Figure 5. Immunohistochemical expression of RunX2. A) Anorganic bovine bone with autograft (micropolymer peroxidase-conjugated, original magnification x20) and B) freeze-dried bone allograft with autograft (micropolymer peroxidase-conjugated, original magnification x10).



REFERENCES

Alayan, J., Vaquette, C., Farah, C. & Ivanovski, S. (2016) A histomorphometric assessment of collagen-stabilized anorganic bovine bone mineral in maxillary sinus augmentation – a prospective clinical trial. *Clinical Oral Implants Research* **27**: 850–858.

Avila, G., Neiva, R., Misch, C.E., Galindo-Moreno, P., Benavides, E., Rudek, I. & Wang, H.L. (2010a) Clinical and histologic outcomes after the use of a novel allograft for maxillary sinus augmentation: a case series. *Implant Dentistry* **19**: 330–41.

Avila, G., Wang, H.L., Galindo-Moreno, P., Misch, C.E., Bagramian, R.A., Rudek, I., Benavides, E., Moreno-Riestra, I., Braun, T. & Neiva, R. (2010b) The influence of the bucco-palatal distance on sinus augmentation outcomes. *Journal of Periodontology* **81**: 1041–50.

Beitlitum, I., Artzi, Z. & Nemcovsky, C.E. (2010) Clinical evaluation of particulate allogeneic with and without autogenous bone grafts and resorbable collagen membranes for bone augmentation of atrophic alveolar ridges. *Clinical Oral Implants Research* **21**: 1242–1250.

Boeck-Neto, R.J., Artese, L., Piattelli, A., Shibli, J.A., Perrotti, V., Piccirilli, M. & Marcantonio, E., Jr. (2009) VEGF and MVD expression in sinus augmentation with autologous bone and several graft materials. *Oral Diseases* **15**: 148–54.

Boyne, P.J. (1973) Implants and transplants: review of recent research in this area of oral surgery. *Journal of the American Dental Association* **87**: 1074–1080.

Busenlechner, D., Huber, C.D., Vasak, C., Dobsak, A., Gruber, R. & Watzek, G. (2009) Sinus augmentation analysis revised: the gradient of graft consolidation. *Clinical Oral Implants Research* **20**: 1078–1083.

Corbella, S., Taschieri, S., Weinstein, R. & Del Fabbro, M. (2016) Histomorphometric outcomes after lateral sinus floor elevation procedure: a systematic review of the literature and meta-analysis. *Clinical Oral Implants Research* **27**: 1106–1122.

Danesh-Sani, S.A., Engebretson, S.P. & Janal, M.N. (2016) Histomorphometric results of different grafting materials and effect of healing time on bone maturation after sinus floor augmentation: a systematic review and meta-analysis. *Journal of*

Periodontal Research n/a-n/a.

Davies, J.E. (2003) Understanding peri-implant endosseous healing. *Journal of Dental Education* **67**: 932–949.

Degidi, M., Artese, L., Rubini, C., Perrotti, V., Iezzi, G. & Piattelli, A. (2007) Microvessel density in sinus augmentation procedures using anorganic bovine bone and autologous bone: 3 months results. *Implant Dentistry* **16**: 317–325.

Froum, S.J., Wallace, S.S., Elian, N., Cho, S.C. & Tarnow, D.P. (2006) Comparison of mineralized cancellous bone allograft (Puros) and anorganic bovine bone matrix (Bio-Oss) for sinus augmentation: histomorphometry at 26 to 32 weeks after grafting. *International Journal of Periodontics and Restorative Dentistry* **26**: 543–51.

Galindo, M., Pratap, J., Young, D.W., Hovhannisyan, H., Im, H.-J., Choi, J.-Y., Lian, J.B., Stein, J.L., Stein, G.S. & Wijnen, A.J. van (2005) The Bone-specific Expression of Runx2 Oscillates during the Cell Cycle to Support a G1-related Antiproliferative Function in Osteoblasts. *Journal of Biological Chemistry* **280**: 20274–20285.

Galindo-Moreno, P., Avila, G., Fernandez-Barbero, J.E., Aguilar, M., Sanchez-Fernandez, E., Cutando, A. & Wang, H.L. (2007) Evaluation of sinus floor elevation using a composite bone graft mixture. *Clinical Oral Implants Research* **18**: 376–82.

Galindo-Moreno, P., Hernández-Cortés, P., Aneiros-Fernández, J., Camara, M., Mesa, F., Wallace, S. & O'Valle, F. (2014) Morphological evidences of Bio-Oss® colonization by CD44-positive cells. *Clinical Oral Implants Research* **25**: 366–371.

Galindo-Moreno, P., Hernández-Cortés, P., Mesa, F., Carranza, N., Juodzbalys, G., Aguilar, M. & O'Valle, F. (2013) Slow resorption of anorganic bovine bone by osteoclasts in maxillary sinus augmentation. *Clinical Implant Dentistry and Related Research* **15**: 858–866.

Galindo-Moreno, P., Hernandez-Cortes, P., Padial-Molina, M., Vizoso, M.L., Crespo-Lora, V. & O'Valle, F. (2015) Immunohistochemical osteopontin expression in bone xenograft in clinical series of maxillary sinus lift. *Journal of Oral Science & Rehabilitation* **1**: 42–50.

Galindo-Moreno, P., Moreno-Riestra, I., Avila, G., Padial-Molina, M., Paya, J.A., Wang, H.L. & O'Valle, F. (2011) Effect of anorganic bovine bone to autogenous

cortical bone ratio upon bone remodeling patterns following maxillary sinus augmentation. *Clinical Oral Implants Research* **22**: 857–64.

Galindo-Moreno, P., Moreno-Riestra, I., Ávila-Ortiz, G., Padial-Molina, M., Gallas-Torreira, M., Sánchez-Fernández, E., Mesa, F., Wang, H.-L. & O'Valle, F. (2012) Predictive factors for maxillary sinus augmentation outcomes: a case series analysis. *Implant Dentistry* **21**: 433–440.

Galindo-Moreno, P., Padial-Molina, M., Fernandez-Barbero, J.E., Mesa, F., Rodríguez-Martínez, D. & O'Valle, F. (2010) Optimal microvessel density from composite graft of autogenous maxillary cortical bone and anorganic bovine bone in sinus augmentation: influence of clinical variables. *Clinical Oral Implants Research* **21**: 221–227.

Gapski, R., Misch, C., Stapleton, D., Mullins, S., Cobb, C., Vansanthan, A. & Reissner, M. (2008) Histological, histomorphometric, and radiographic evaluation of a sinus augmentation with a new bone allograft: a clinical case report. *Implant Dentistry* **17**: 430–438.

Hallman, M., Sennerby, L. & Lundgren, S. (2002) A clinical and histologic evaluation of implant integration in the posterior maxilla after sinus floor augmentation with autogenous bone, bovine hydroxyapatite, or a 20:80 mixture. *International Journal of Oral and Maxillofacial Implants* **17**: 635–43.

Handschel, J., Simonowska, M., Naujoks, C., Depprich, R.A., Ommerborn, M.A., Meyer, U. & Kübler, N.R. (2009) A histomorphometric meta-analysis of sinus elevation with various grafting materials. *Head & Face Medicine* **5**: 12.

Hong, I.-S. & Kang, K.-S. (2013) The Effects of Hedgehog on the RNA-Binding Protein Msi1 in the Proliferation and Apoptosis of Mesenchymal Stem Cells. *PLOS ONE* **8**: e56496.

Huang, Y.-C., Kaigler, D., Rice, K.G., Krebsbach, P.H. & Mooney, D.J. (2005) Combined angiogenic and osteogenic factor delivery enhances bone marrow stromal cell-driven bone regeneration. *Journal of Bone and Mineral Research* **20**: 848–857.

Jensen, T., Schou, S., Stavropoulos, A., Terheyden, H. & Holmstrup, P. (2012) Maxillary sinus floor augmentation with Bio-Oss or Bio-Oss mixed with autogenous

bone as graft: a systematic review. *Clinical Oral Implants Research* **23**: 263–273.

Kaigler, D., Avila-Ortiz, G., Travan, S., Taut, A.D., Padial-Molina, M., Rudek, I., Wang, F., Lanis, A. & Giannobile, W.V. (2015) Bone engineering of maxillary sinus bone deficiencies using enriched CD90+ stem cell therapy: a randomized clinical trial. *Journal of Bone and Mineral Research* **30**: 1206–1216.

Kaneko, Y., Sakakibara, S., Imai, T., Suzuki, A., Nakamura, Y., Sawamoto, K., Ogawa, Y., Toyama, Y., Miyata, T. & Okano, H. (2000) Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Developmental Neuroscience* **22**: 139–153.

Lee, D.-W., Pi, S.-H., Lee, S.-K. & Kim, E.-C. (2009) Comparative histomorphometric analysis of extraction sockets healing implanted with bovine xenografts, irradiated cancellous allografts, and solvent-dehydrated allografts in humans. *The International Journal of Oral & Maxillofacial Implants* **24**: 609–615.

Lundgren, S., Cricchio, G., Hallman, M., Jungner, M., Rasmusson, L. & Sennerby, L. (2017) Sinus floor elevation procedures to enable implant placement and integration: techniques, biological aspects and clinical outcomes. *Periodontology 2000* **73**: 103–120.

Misch, C.E., Perel, M.L., Wang, H.-L., Sammartino, G., Galindo-Moreno, P., Trisi, P., Steigmann, M., Rebaudi, A., Palti, A., Pikos, M.A., Schwartz-Arad, D., Choukroun, J., Gutierrez-Perez, J.-L., Marenzi, G. & Valavanis, D.K. (2008) Implant success, survival, and failure: the International Congress of Oral Implantologists (ICOI) Pisa Consensus Conference. *Implant Dentistry* **17**: 5–15.

Monje, A., O'Valle, F., Monje-Gil, F., Ortega-Oller, I., Mesa, F., Wang, H.-L. & Galindo-Moreno, P. (2017) Cellular, vascular, and histomorphometric outcomes of solvent-dehydrated vs freeze-dried allogeneic graft for maxillary sinus augmentation: a randomized case series. *The International Journal of Oral & Maxillofacial Implants* **32**: 121–127.

Mordenfeld, A., Johansson, C.B., Albrektsson, T. & Hallman, M. (2014) A randomized and controlled clinical trial of two different compositions of deproteinized bovine bone and autogenous bone used for lateral ridge augmentation. *Clinical Oral*

Implants Research **25**: 310–320.

Nagata, T., Kanno, R., Kurihara, Y., Uesugi, S., Imai, T., Sakakibara, S., Okano, H. & Katahira, M. (1999) Structure, backbone dynamics and interactions with RNA of the C-terminal RNA-binding domain of a mouse neural RNA-binding protein, Musashi1. *Journal of Molecular Biology* **287**: 315–330.

Okano, H., Imai, T. & Okabe, M. (2002) Musashi: a translational regulator of cell fate. *Journal of Cell Science* **115**: 1355–1359.

Okano, H., Kawahara, H., Toriya, M., Nakao, K., Shibata, S. & Imai, T. (2005) Function of RNA-binding protein Musashi-1 in stem cells. *Experimental Cell Research* **306**: 349–356.

Orsini, G., Traini, T., Scarano, A., Degidi, M., Perrotti, V., Piccirilli, M. & Piattelli, A. (2005) Maxillary sinus augmentation with Bio-Oss® particles: A light, scanning, and transmission electron microscopy study in man. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **74B**: 448–457.

Padial-Molina, M., O'Valle, F., Lanis, A., Mesa, F., Dohan Ehrenfest, D.M., Wang, H.-L. & Galindo-Moreno, P. (2015) Clinical application of mesenchymal stem cells and novel supportive therapies for oral bone regeneration. *BioMed Research International* **2015**: 341327.

Parfitt, A.M. (2000) The mechanism of coupling: a role for the vasculature. *Bone* **26**: 319–323.

Roberts, T.T. & Rosenbaum, A.J. (2012) Bone grafts, bone substitutes and orthobiologics. *Organogenesis* **8**: 114–124.

Schmitt, C.M., Doering, H., Schmidt, T., Lutz, R., Neukam, F.W. & Schlegel, K.A. (2013) Histological results after maxillary sinus augmentation with Straumann® BoneCeramic, Bio-Oss®, Puros®, and autologous bone. A randomized controlled clinical trial. *Clinical Oral Implants Research* **24**: 576–585.

Schmitt, C.M., Moest, T., Lutz, R., Neukam, F.W. & Schlegel, K.A. (2015) Anorganic bovine bone (ABB) vs. autologous bone (AB) plus ABB in maxillary sinus grafting. A prospective non-randomized clinical and histomorphometrical trial. *Clinical Oral Implants Research* **26**: 1043–1050.

Sureban, S.M., May, R., George, R.J., Dieckgraefe, B.K., McLeod, H.L., Ramalingam, S., Bishnupuri, K.S., Natarajan, G., Anant, S. & Houchen, C.W. (2008) Knockdown of RNA binding protein musashi-1 leads to tumor regression in vivo. *Gastroenterology* **134**: 1448–1458.

Thorwarth, M., Schlegel, K.A., Wehrhan, F., Srour, S. & Schultze-Mosgau, S. (2006) Acceleration of de novo bone formation following application of autogenous bone to particulated anorganic bovine material in vivo. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics* **101**: 309–16.

Traini, T., Piattelli, A., Caputi, S., Degidi, M., Mangano, C., Scarano, A., Perrotti, V. & Iezzi, G. (2015) Regeneration of human bone using different bone substitute biomaterials. *Clinical Implant Dentistry and Related Research* **17**: 150–162.

Velasco-Torres, M., Padial-Molina, M., Alarcón, J.A., O'Valle, F., Catena, A. & Galindo-Moreno, P. (2016) Maxillary sinus dimensions with respect to the posterior superior alveolar artery decrease with tooth loss. *Implant Dentistry* **25**: 464–470.

Velasco-Torres, M., Padial-Molina, M., Avila-Ortiz, G., García-Delgado, R., O'Valle, F., Catena, A. & Galindo-Moreno, P. (2017) Maxillary sinus dimensions decrease as age and tooth loss increase. *Implant Dentistry* **26**: 288–295.

Zerbo, I.R., Bronckers, A.L., de Lange, G. & Burger, E.H. (2005) Localisation of osteogenic and osteoclastic cells in porous beta-tricalcium phosphate particles used for human maxillary sinus floor elevation. *Biomaterials* **26**: 1445–51.

Zerbo, I.R., de Lange, G.L., Joldersma, M., Bronckers, A.L.J.J. & Burger, E.H. (2003) Fate of monocortical bone blocks grafted in the human maxilla: a histological and histomorphometric study. *Clinical Oral Implants Research* **14**: 759–766.