Nanocrystalline Hydroxyapatite Bone Substitute Leads to Sufficient Bone Tissue Formation Already after 3 Months: Histological and Histomorphometrical Analysis 3 and 6 Months following Human Sinus Cavity Augmentation

Shahram Ghanaati, MD, DDS;[*,†] Mike Barbeck, MSc;[†] Ines Willershausen, DDS;[‡] Benjamin Thimm, PhD;[§] Stefan Stuebinger, DDS;[¶] Tadas Korzinskas, DDS;[*] Karina Obreja;[*] Constantin Landes, MD, DDS, PhD;[*] Charles J. Kirkpatrick, MD, PhD;[†] Robert A. Sader, MD, DDS, PhD[*]

ABSTRACT

Purpose: In this study the de novo bone formation capacity of a nanocrystalline hydroxyapatite bone substitute was assessed 3 and 6 months after its insertion into the human sinus cavity.

Materials and Methods: Sinus cavity augmentation was performed in a total of 14 patients ($n = 7$ implantation after 3 months; $n = 7$ implantation after 6 months) with severely atrophic maxillary bone. The specimens obtained after 3 and 6 months were analyzed histologically and histomorphometrically with special focus on bone metabolism within the residual bone and the augmented region.

Results: This study revealed that bone tissue formation started from the bone-biomaterial-interface and was directed into the most cranial parts of the augmented region. There was no statistically significant difference in new bone formation after 3 and 6 months (24.89 ± 10.22% vs 31.29 ± 2.29%), respectively.

Conclusions: Within the limits of the present study and according to previously published data, implant insertion in regions augmented with this bone substitute material could be considered already after 3 months. Further clinical studies with bone substitute materials are necessary to validate these findings.

KEY WORDS: NanoBone, nanocrystalline hydroxyapatite, sinus floor augmentation

INTRODUCTION

The sinus lift procedure is an important augmentation technique in order to increase the vertical and horizontal bone height in atrophic maxillary bones prior to dental implant insertion. Ever since its introduction, this technique has markedly improved, although a consensus on the ideal grafting material has yet to be found.[1,2]

Autologous bone was the first material used for augmentation of the sinus floor[2] and is still regarded as the "gold standard" grafting material. The osteogenic, osteoconductive, and osteoinductive properties are considered to be the main advantage of autologous bone grafts over other bone substitute materials.[3] Moreover,

[*]Department for Oral, Cranio-Maxillofacial and Facial Plastic Surgery, Medical Center of the Goethe University Frankfurt, Frankfurt am Main, Germany; [†]Institute of Pathology, REPAIR-Lab, University Medical Center, Johannes Gutenberg University Mainz, Germany; [‡]Institute for Dental Material Sciences and Technology, University Medical Center of the Johannes Gutenberg University Mainz, Germany; [§]Institute for Biomechanics, ETH Zurich, Switzerland; [¶]Center for Applied Biotechnology and Molecular Medicine, Zurich, Switzerland

Reprint requests: Dr. Shahram Ghanaati, Clinic for Maxillofacial and Plastic Surgery, Johann Wolfgang Goethe University, Frankfurt/Main, Germany; e-mail: Ghanaati@uni-mainz.de

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there is evidence that augmentation sites, which are grafted with autologous bone grafts, show a more rapid bone formation.[4] The main drawbacks of autologous bone grafts are the increased resorption rate, the need for general anesthesia, and the additional surgical site required for graft extraction, which is associated with increased patient morbidity.[5,6]

Allo- and xenografts account for another source of grafting materials and are widely used and well documented.[7] Studies even report a higher implant survival rate within xenografts than with autologous bone.[8,9] When comparing allografts with autologous bone grafts on a histological basis, Froum and colleagues[10] reported that less vital bone is found for xenograft implantation sites as opposed to autografts (12.44 and 28.25%, respectively) after a healing period of 6 months. Processing techniques such as lyophilization, irradiation, or freeze drying, which are necessary to remove all immunogenic properties from allo- and xenograft, are believed to negatively influence the osteoregenerative qualities of these materials.

A large number of synthetic bone substitutes are available, for example, alpha- and beta-tricalcium phosphate (β-TCP), biphasic calcium phosphate ceramics, and hydroxyapatite (HA) exist, and these materials might be good alternatives to both allo- and xenograft, as well as autologous bone grafts. The use of these materials is intended to minimize the disadvantages mentioned previously. HA ($Ca_5[PO_4]_3OH$), is a calcium phosphate-based material, which makes up approximately 55% of the bone weight in vertebrates.[11] On account of its osteoconductive qualities, it is widely applied in the field of orthopedic and oral surgery.[3,12] As an essential trace element, silicon is involved in both connective tissue formation and osteoblast proliferation. Further, it is believed to positively influence human bone mineralization and calcification processes.[13,14] The fully synthetic bone substitute, NanoBone (Artoss, Rostock, Germany), which was applied in these clinical studies, is basically a nanocrystalline HA embedded in a silica gel matrix, achieved by means of specific sol-gel techniques.[14,15] Features such as interconnecting pores on the nanoscale, the open SiOH or SiO groups of polysilicic acid, its large internal surface, and the high porosity of this biomaterial are all related to the calcification processes observed within the implantation bed.[14,15] While the HA component is responsible for NanoBone's osteoconductive properties, the silica component is believed to induce connective tissue formation, osteoblast proliferation, bone matrix mineralization, and calcification, thus combining osteoconductive and osteoinductive properties.[13,15–18] This phenomenon is associated with the rearrangement of the silica matrix, which could be observed in vivo.[17,18]

Based on the observations gained from animal experiments, several clinical studies were carried out, thus confirming the properties of this nanocrystalline HA in clinical applications.[16,17,19,20] Our group has previously assessed the tissue reaction to the nanocrystalline HA in human sinus cavities.[19] Six months after implantation, the presence of newly formed bone, starting from the bone-biomaterial interface, was observed. The cellular degradation of the nanocrystalline HA was verified by the presence of tartrate-resistant acid phosphatase (TRAP)-positive mono- and multinucleated cells.[19] Canullo and Dellavia[20] compared the capacity of de novo bone formation induced by nanocrystalline HA after 3 and 6 months following insertion into human maxillary sinus. The latter authors observed a significant difference in new bone formation between 3 and 6 months (8% new bone after 3 months and 48% after 6 months), respectively. Based on these findings, they claimed that a healing period of 6 months prior to implantation should be preferred to 3 months. In a subsequent study, the authors reevaluated de novo bone formation 3 months after insertion of the same nanocrystalline HA.[21] In contrast to the earlier finding, they were able to report about 20% newly formed bone after 3 months.[21]

From these discrepant reports regarding the amount of new bone formation within the sinus cavity 3 months after implantation of this nanocrystalline HA, it became evident that a reassessment of the capacity for de novo bone formation was necessary in a similar study setting. In the present clinical investigation, the tissue reaction to this nanocrystalline bone substitute material was tested on a histological level. After sinus floor elevation and material biopsy harvesting 3 and 6 months after implantation according to a previously established histomorphometrical protocol.[19]

MATERIALS AND METHODS

Bone Grafting Substitute

NanoBone, a fully synthetic bone substitute material is composed of HA crystallites with an average size of

60 nm, which are embedded in a matrix of structured silica gel.[14,15] It is produced by means of the sol-gel technique, with temperatures below 700°C, so that no sintering of the nanocrystalline HA takes place.[14,15] In the transition process from sol into gel, a loose connection of the HA crystals with the SiO_2 molecules takes place, resulting in a nanoporously structured bone substitute. The silica gel is characterized by numerous open bonds, which are responsible for an internal surface of up to 84 m^2/g in size.[14,15] The pore sizes found within the silica gel range within diameters from 5 to 50 nm.[14,15] Macroscopically, a single NanoBone granule is shaped like a fir cone with an average length of 2 mm and an average diameter of 0.6 mm with a porosity of 60–80%.[14,15]

Study Design in Humans

A total of 14 fully or partially edentulous patients (eight women, six men) from the Department of Oral, Cranio-Maxillofacial and Facial Plastic Surgery, Frankfurt am Main, underwent sinus elevation procedure. The study group had an average age of 64 (52–79). The study was approved by the Ethics Commission of the University of Frankfurt am Main and was carried out in accordance with the Fifth revision of the World Medical Association Declaration of 2000. Informed consent for the sinus augmentation procedure and the present study was achieved prior to surgery. Patients were in good general health with no contraindications against oral surgical interventions. Prior to surgery, basic anamnestic data were recorded and included medical history, smoking habits, as well as an examination of the oral cavity. All of the participants had to be free of any sinus pathology and displayed a reduced height of the alveolar crest (less than 5 mm, with a mean of 2.07 ± 0.92 mm) in the prospective implant site.

The surgical procedure was carried out either under general (nine patients) or local anesthesia (five patients). The lateral access was performed by a vestibular standard incision and development of a mucoperiosteal flap with a vestibular and superior basis. The antrostomy was performed utilizing the Piezosurgery® device (Mectron, Carasco, Italy). The sinus cavities showed no abnormal septa formations. In three cases, shallow underwood septa formations could be observed. The size and the configuration of the septa did not interfere with the surgery. No serious complications occurred during the sinus lift surgery. Two Schneiderian membranes perforation sized 1×1.5 mm were covered with a collagen membrane (Bio-Gide®, Geistlich Söhne AG, Wolhusen, Switzerland). None of the patients developed a chronic sinusitis condition following the procedure. All other Schneiderian membranes have been elevated successfully without tearing. The prepared cavity was augmented with the synthetic nanostructured HA graft material. The biomaterial was mixed with blood gained from the surgical site and was densely packed into the cavity. No additional autogenous bone blocks or chips were used. An additional covering of the surgical site was performed by means of a collagen membrane (Bio-Gide). Primary wound closure was accomplished with resorbable tension-free single sutures. Postoperatively, Augmentan 875/125 was started intraoperatively via intravenous application and prescribed as oral medication for 10 days post op. Chlorxehydine 0.2% mouthrinse was recommended three times a day for 10 days. Ibuprofen 400 mg was the standard analgetic agent.

During second stage surgery of the dental implant placement (CAMLOG® Screw Line, CAMLOG Biotechnologies, Basel, Switzerland) after 3 and 6 months, 14 cylinder-shaped bone biopsies (seven biopsies per time point) were taken from the augmented maxillary region through a crestal approach using trephine burs (3 mm). Altogether, 32 Screw Line Promote Implants sized 4.3 and 5 mm in diameter and 11 mm length have been inserted in the augmented areas.

Six months after implant placement, second stage surgery was performed. The implants were exposed by performing the roll flap technique and a healing abutment was incorporated.

Tissue Preparation and Histology for Human Bone Biopsies

For analysis of bone-biomaterial interaction, a total of 14 available human biopsies, harvested from the maxillary sinus, were analyzed histologically as previously described.[19] These biopsies were collected after 90 ($n = 7$) or 180 days ($n = 7$) after implantation. The bone biopsies were fixed in 4% neutral buffered formalin for 24 hours, decalcified in Tris-buffered 10% EDTA (Carl Roth, Karlsruhe, Germany) at 37°C for 4 days, and then subsequently dehydrated in a series of increasing alcohol concentrations followed by xylol. Paraffin embedding was performed and sections of 4 µm thickness in the longitudinal plane (along the sagittal axis) of the biopsy were cut with a microtome (Leica, Wetzlar, Germany). For each biopsy, three consecutive sections were

collected in the central portion of bone cores. The sections were then stained for light microscopy as follows. The first and second sections were stained with hematoxylin and eosin and Masson – Goldner's trichrome and counterstained with Weigert's iron hematoxylin, respectively.[19] The third section was used to identify osteoclasts by histochemical staining for TRAP according to previously described methods.[19,21]

Histological Analysis

Histopathological evaluation was conducted as previously described.[19,22–24] Briefly, the outcome of the tissue-biomaterial interaction in the specimens was evaluated by examination of a total implantation bed and its peri-implant tissue. The extent of the inflammatory response provoked by the biomaterial was described by using the following characteristics: vascularization, multinucleated giant cells, and TRAP-negative and TRAP-positive osteoclast-like cells. Other parameters such as fibrosis, hemorrhage, necrosis, presence of neutrophils, lymphocytes, plasma cells, and macrophages, were only described when being severely present within the implantation bed. Microphotographs were taken using a digital camera and a digital sight control unit (Nikon, Tokyo, Japan).

Histomorphometry in Human Sinus Biopsies

The histomorphometric analysis was performed using the software *NIS-Elements* (Nikon, Tokyo, Japan) according to the manufacturer's instructions as previously described.[19,22,23] Using the software NIS-Elements and the implemented "annotations and measurements" tool, the number and percentage of vessels, as well as the number of TRAP-negative and TRAP-positive multinucleated giant cells were quantified within the augmented regions as well as within the residual bone segments of the biopsies, respectively. These parameters were related to 1 mm^2 of each corresponding specimen area. Additionally, the extent of newly formed bone tissue, the remaining volume of bone substitute and the amount of the connective tissue within the augmented region were measured and calculated as the percentage fraction of the total sum of these three mentioned components. A statistical analysis was performed, comparing the area of the biomaterial granules, the newly formed bone tissue, as well as the amount of connective tissue within the augmented region at each time point. A separate analysis was conducted which compared the data of these three components within the two implantation beds at the two study time points.

Statistical Analysis

Quantitative data are presented as mean ± standard deviation, and a one-way univariate analysis of variance followed by least significant difference post hoc assessment was applied to compare groups using the SPSS 16.0.1 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant if p-values were less than .05, and highly significant if p-values were less than 0.01. The SigmaPlot 11.0 software (SigmaPlot, Systat Software Inc., Erkrath, Germany) was used for plotting graphs.

Histological and Histomorphometric Results for Human Sinus Biopsies

Analysis of the Tissue Reaction after 3 Months. At day 90 after implantation, within the analyzed augmented fraction, the biomaterial was well integrated in the surrounding new tissue composed of connective tissues and bone trabeculae (Figure 1A). Both TRAP-positive and TRAP-negative multinucleated giant cells were involved in the cellular degradation of the bone substitutes. These cells originated from the connective tissue within the implantation bed and were located on the bone substitute surface (see Figure 1B). Newly formed bone was observed in some samples ($n = 3$) in the caudal parts of the biopsies, sparing the most cranial regions of the biopsy (see Figure 1C). In the majority of the analyzed samples ($n = 5$), however, new bone formation was observed within all parts of the biopsy (see Figure 1D).

The histomorphometrical analysis of the percental distribution of new bone tissue, bone substitute material, and connective tissue revealed that within the augmented region, the amount of the used bone substitute, the newly formed bone, as well as the connective tissue were 29.28 ± 12.24%, 24.89 ± 10.22%, and 45.81 ± 20.52%, respectively (Figure 3A).

The histomorphometrical analysis of vessel density and distribution of multinucleated giant cells was performed by relating the number of these parameters to 1 mm^2 (number of vessels/mm^2 or number of cells/mm^2) of the implantation bed. In this newly formed tissue within the augmented fraction vessel density, the amount of TRAP-positive multinucleated giant cells and the total amount of multinucleated giant cells was significantly higher when compared with the analyzed

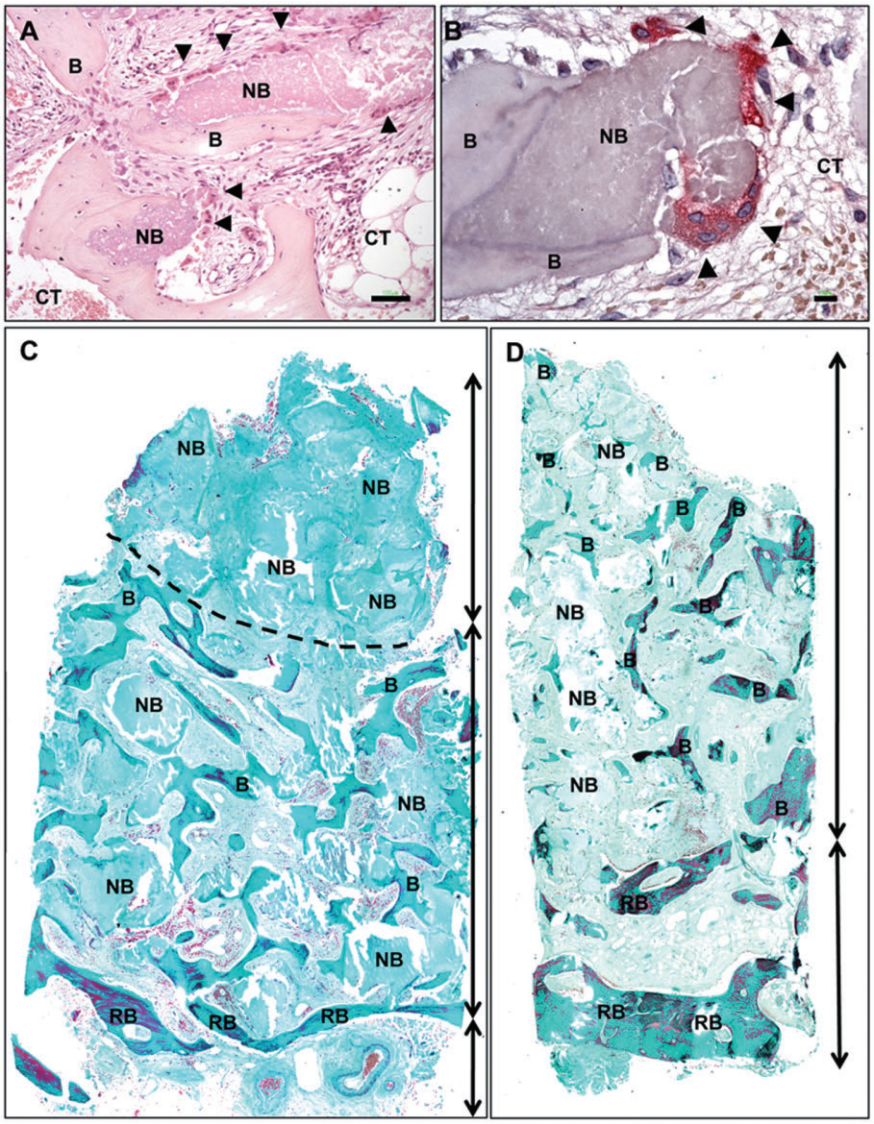

Figure 1 The figure shows NanoBone's (NB's) integration within the augmentation area 3 months after augmentation. *A*, Shows new bone (B) formation on the surface of NB and its cellular degradation by means of multinucleated giant cells (*arrow heads*) within the bone substitute surrounding connective tissue (CT) (H&E staining, ×200 magnification, scale bar: 100 mm). *B*) Shows multinucleated giant cells (*arrow heads*) within the augmentation area. These cells are located at the interface between CT and NB while sparing the interface between bone (B) and the bone substitute material (TRAP staining, ×600 magnification, scale bar: 100 mm). *C*) Demonstrates that in some biopsies, new bone formation around NB did not reach the most cranial aspects of the biopsy (*broken line*) (total scan, Masson – Goldner stain, ×100 magnification, scale bar: 100 mm). *D*) Shows that in some other samples, new bone formation (B) reaches all part of the biopsy (total scan, Masson – Goldner stain, ×100 magnification, scale bar: 100 mm).

residual bone fractions within this group ($p > .01$) (see Figure 3B). Interestingly, the amount of TRAP-negative multinucleated giant cells was significantly lower in the augmented region when compared with the residual bone area ($p > .01$) (see Figure 3B).

Analysis of the Tissue Reaction after 6 Months. At day 180 after implantation, the biomaterial granules within the analyzed augmented fraction were well integrated in a surrounding newly built tissue composed of connective tissue and bone trabeculae (Figure 2A). At this time point, TRAP-positive as well as TRAP-negative multinucleated giant cells were still involved in bone substitute degradation (see Figure 2B). Compared with day 90, newly formed bone was observed within all parts of all analyzed biopsies in all of the samples (see Figure 2C).

The histomorphometrical analysis of the percentage distribution of new bone tissue, bone substitute material, and connective tissue at this time point revealed that within the augmented region, the amount of the

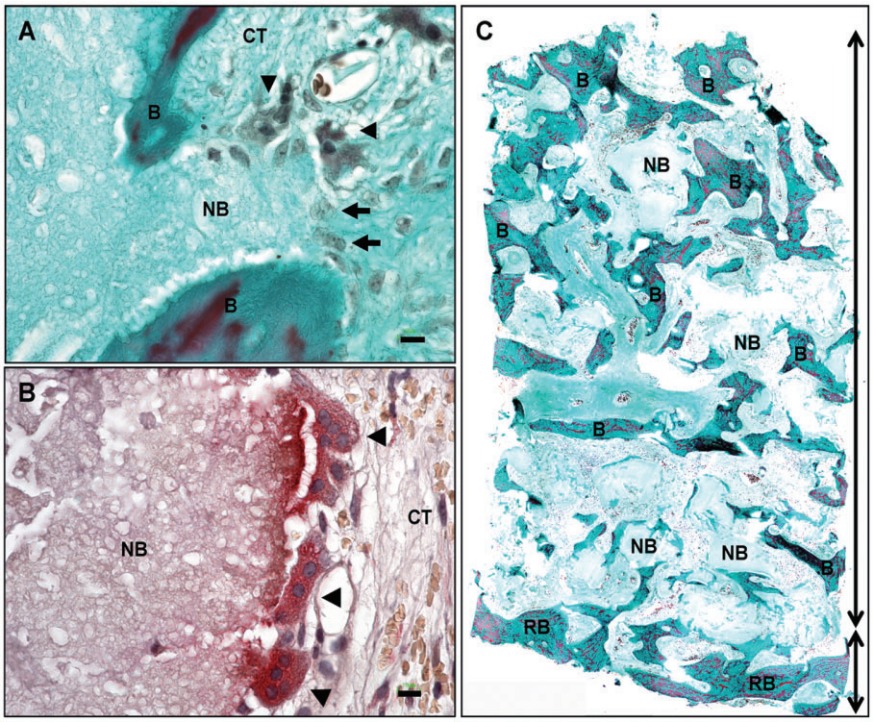

Figure 2 The figure illustrates NanoBone (NB) integration within the implantation bed 6 months after implantation. *A*) Shows bone tissue maturation within the augmentation area (*red areas*; *arrow heads*) within the bone trabeculae (B) (Masson – Goldner stain, ×600 magnification, scale bar: 100 mm). *B*) Displays the ongoing degradation of NB by TRAP-positive multinucleated giant cells (*arrow heads*) (TRAP staining, ×600 magnification, scale bar: 100 mm). *C*) Shows homogenous new bone formation within all parts of the biopsy (total scan, Masson – Goldner stain, ×100 magnification, scale bar: 100 mm). CT = connective tissue.

used bone substitute, the newly formed bone, and the connective tissue were 16.74 ± 1.72%, 31.29 ± 2.29%, and 51.97 ± 3.59%, respectively.

The histomorphometrical analysis revealed that the vessel density, the amount of TRAP-positive multinucleated giant cells, and the total number of the multinucleated giant cells at this time point were also significantly higher when compared with the analyzed residual bone fractions of this group ($p > .01$) (see Figure 3C). As observed for the 3 months group, the amount of TRAP-negative multinucleated giant cells was significantly lower in the augmented region when compared with the residual bone ($p > .01$) (see Figure 3C).

Comparative Histomorphometrical Analysis of Tissue Distribution (3 vs 6 Months after Implantation). Within the augmented region, the bone substitute underwent a statistically relevant degradation within the time period of 3 and 6 months after implantation (29.29 ± 12.25% vs 16.74 ± 1.72%; $p > .05$) (see Figure 3D). This degradation positively correlated with newly formed bone tissue and the amount of connective tissue within the augmentation bed (70.71 ± 15.38% vs 83.26 ± 1.79%; $p > .05$) (see Figure 3D). However, no statistically significant correlation could be found between bone substitute material degradation and new bone formation or connective tissue ingrowth alone.

DISCUSSION

Understanding tissue reaction evoked by the integration of a bone substitute and its subsequent degradation is crucial in order to predict its clinical performance. In this clinical study, the integration and the degradation of a nanocrystalline HA-based bone substitute was evaluated by means of histological and histomorphometrical analysis. The histological data showed that after 3 months, bone regeneration had reached almost two-thirds of the augmented region. In all biopsies obtained after 6 months, newly formed bone was detectable, even at the most cranial parts of the biopsy. These data underline the osteoconductive characteristics of this bone substitute, inducing a time-dependent bone regeneration, which is initiated from the residual bone.

The histomorphometrical analysis revealed a significantly higher vessel density within the augmented

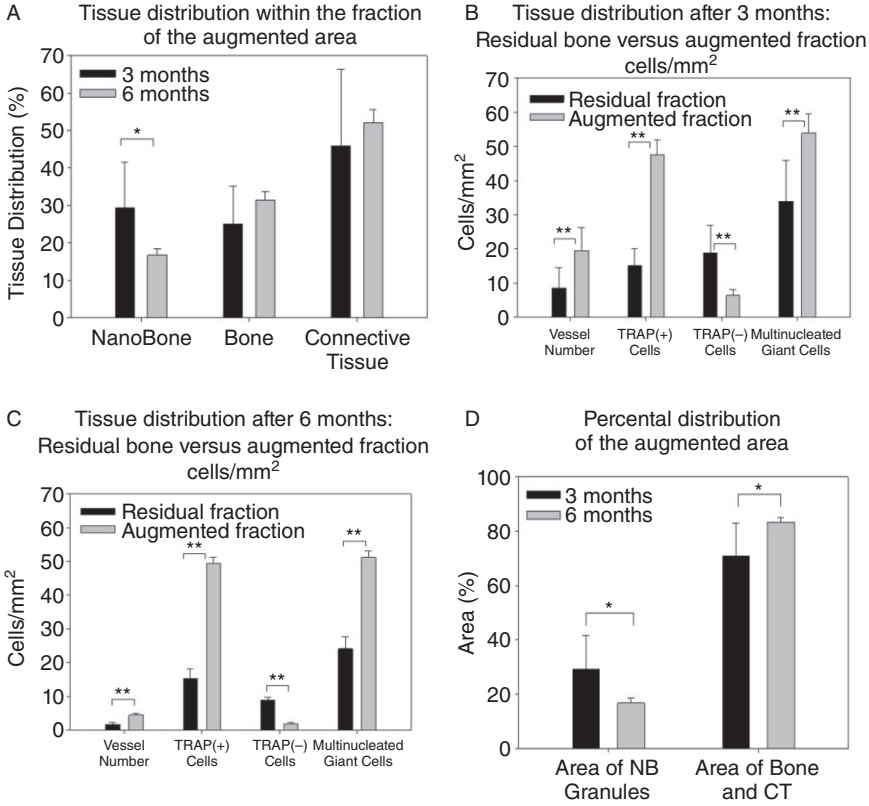

Figure 3 The figure displays the histomorphometrical analysis of the different components within the implantation bed. *A*) Shows the percentage distribution of bone tissue, bone substitute material, and connective tissue (CT) 3 and 6 months after implantation. *B*) Demonstrates the amount of vessels, TRAP-negative, and TRAP-positive multinucleated giant cells within the implantation bed after 3 months. *C*) Illustrates the same parameters 6 months after implantation, for example, the amount of vessels, TRAP-negative, and TRAP-positive multinucleated giant cells within the implantation bed. *D*) Shows the correlation between bone substitute's degradation and bone and CT enrichment within the augmentation area. A statistical degradation of the bone substitute material between 3 and 6 months became apparent (29.29 ± 12.25% vs 16.74 ± 1.72%; $p < .05$), while at the same time the area of bone and CT statistically increased (70.71 ± 15.38% vs 83.26 ± 1.79%; $p < .05$). $^{*}p < .05$; $^{**}p < .01$.

region when compared with residual bone. This underlines the importance of a sufficient blood supply as a vital factor for successful bone regeneration. Furthermore, the high blood vessel density is in keeping with the increased metabolism within the augmented areas, which is necessary for chemotaxis of inflammatory cells involved in biomaterial degradation. This high vascularization might be the reason for the enrichment in the implantation bed with TRAP-positive multinucleated giant cells, known for their ability to degrade ceramic-based bone substitute materials. These cells, also called "osteoclast-like cells," markedly contribute to biomaterial degradation and dissolution. The presence of TRAP-positive multinucleated giant cells within the human sinus cavities correlates with previously performed human studies.[19] The significantly higher presence of TRAP-positive multinucleated giant cells within the augmented region as opposed to the residual bone leads to the assumption that the bone remodelling process of the residual bone tissue is slower than that found in the augmented region.

The histomorphometrical analysis of the histological specimens at the two time points showed that the present bone substitute undergoes a process of continuous degradation. These results are in accordance with previously performed preclinical studies, in which a continuous degradation of this material was observed over a period of 6 months in the subcutaneous tissue of Wistar rats[22] or in goat muscle tissue (unpublished data). Accordingly, the biodegradation process of the used material seems to involve cellular resorption rather than an enzymatic degradation. These characteristics emphasize the mechanical stability of this material.

This study revealed that new bone formation within the sinus cavity averaged 24.89 ± 10.22% after 90 days

and 31.29 ± 2.29% after 180 days, respectively. The present study also underlined the fact that biomaterial degradation is not associated with new bone formation alone, but rather with mixed tissue of bone and connective tissue. It is known that the biodegradation of a synthetic bone substitute material and the induced inflammatory response depends on the physicochemical characteristics of the biomaterial. Parameters such as shape, size, and porosity are known to influence the inflammatory response and the degree of new bone formation. However, the interpretation of a lack of correlation between material degradation and new bone formation would be speculative unless other bone substitute materials examined in the same study setting would serve as controls.

Most of the studies on biomaterials applied as sinus grafts focused on histological analysis of bone samples obtained 6 months after bone substitute insertion. In synthetic bone substitute materials, for example, β-TCP-based bone substitutes, the amount of newly formed bone after 8 months is reported to be in the range of 20%,[25] while the use of a biphasic calcium phosphate leads to 27% of new bone formation.[26] In cases in which bovine-origin natural bone mineral (NBM) bone substitutes were used the amount of newly formed bone reaches 39.8 or 40%.[27] Traini and colleagues[28] analyzed a patient collective 9 years after sinus grafting with bovine-based NBM and found the amount of newly formed bone to be around 46%. Furthermore, in cases in which the bovine-based NBM was mixed with autologous bone, the percentage of newly formed bone reached 51%.[29] The results of the present study reveal that after 90 days of implantation the amount of newly formed bone is comparable with values of other synthetic bone substitutes after 6 months.

The results of the present study are, however, not in accordance with the study carried out by Canullo and Dellavia[20] who observed a significant difference in new bone formation between 3 and 6 months (8% new bone after 3 months and 48% after 6 months), respectively. Canullo and Dellavia[20] made use of a human sinus model to determine the capacity of the nanocrystalline HA for de novo bone formation after 3 and 6 months following insertion of the biomaterial. The authors observed a significant difference in new bone formation between 3 and 6 months. Based on these observations, the authors claimed that a healing period of 6 months prior to implantation should be preferred to the earlier time point of 3 months.[20]

In a subsequent study, Canullo and colleagues[21] reevaluated the de novo bone formation properties of this nanocrystalline HA-based bone substitute material, 3 months after insertion into the human sinus cavity. As opposed to their earlier data published in 2009, the most recent results indicate the presence of 20% newly formed bone after 3 months. Those findings are also in accordance with our results, which demonstrate that the amount of newly formed bone is relatively high already at early time points after implantation and that this does not significantly increase when comparing those early values with a time point 6 months after implantation.

These results strongly suggest that a sufficient new bone formation is present already 3 months after augmentation and that the circumstances, which resulted in the values observed by Canullo and Dellavia in 2009 are apparently not reproducible.

The amount of newly formed bone after sinus grafting needed for sufficient, long-lasting osteointegration of dental implants is considered to range between 25 and 35%.[5,30] Tarnow and colleagues[31] stated that bone volume less than 20% presents a higher risk for implant failure. In a sheep model, the biomechanical properties of implants placed in grafted sinus cavities were investigated by Brånemark and colleagues and Haas and colleagues.[32,33] The measured pull out strength ranged between 250 and 500 N, and increased continuously when measured 12 and 26 weeks after implant placement. It is known that implant insertion initiates bone remodelling, thus leading to increased bone formation around the implant.[34,35]

However, the amount of bone at the bone-implant interface does not solely depend on the quality of regenerated bone but also on the implant surface.[36] In the present study, we used implants with a sand-blasted, acid-etched surface, which is known to have a positive osteointegrative effect.[37,38] Nelson and colleagues[39] were able to demonstrate that the early loading of these implants (6–12 weeks after insertion) can be considered, without potentially negative effects on long-term stability. Because of the relatively small patient collective, further clinical studies with other bone substitute materials and other implants have to be performed in order to validate the benefits of early implantation time points.

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