

# Immunohistochemical osteopontin expression in bone xenograft in clinical series of maxillary sinus lift

## Abstract

### Objective

The objectives of this study were to examine osteopontin (OPN) expression in bone and anorganic bovine bone (ABB) in maxillary sinus grafts after six months of healing and to study its relationship to morphological and immunohistochemical results and to patient variables and habits.

### Materials and methods

Forty maxillary sinus lift procedures were performed in 40 consecutive patients. Bone cores were obtained from implant receptor sites at implant placement for histological, morphometric and immunohistochemical studies.

### Results

Histomorphometric analysis found  $32.75 \pm 14.0\%$  vital bone,  $39.49 \pm 17.4\%$  connective tissue, and  $27.75 \pm 21.8\%$  remnant ABB particles. OPN expression was diffuse in 77.5% (31/40) of ABB samples and focal in 22.5% (9/40); it was diffuse in 80% (8/10) of pristine bone samples and focal in 20% (2/10). OPN immunostaining of ABB particles was intense in 45% of maxillary sinus lift biopsies, moderate in 27.5%, mild in 10%, and absent in 17.5%. OPN expression was mainly detected at the interstitial boundary of bone with ABB particles and within osteocyte lacunae and bone canaliculi.

### Conclusion

Immunohistochemical expression of OPN is related to bone remodeling and maturation changes in maxillary sinus lift procedures with ABB xenograft.

### Keywords

Anorganic bovine bone, bone remodeling, intrasinus graft, immunohistochemistry, osteopontin.

## Introduction

Osteopontin (OPN) human gene contains seven exons, spans ~11.1 kb, and maps to the long arm of chromosome 4 (4q13).<sup>1,2</sup> OPN is expressed by a single-copy gene as a ~34 kDa<sup>3</sup> nascent protein that is extensively modified by post-translational events; it is secreted as a noncollagenous acidic bone matrix of single-chain phosphoglycoproteins with diverse functions, including cell-binding activity<sup>4</sup> and angiogenesis.<sup>5</sup> OPN has calcium-binding properties and is expressed by cells in a wide variety of tissues, including bone, tooth and cartilage, and in activated macrophages and lymphocytes.<sup>6</sup>

Data are available on the structure, location and properties of OPN, but the biological function of this protein in bone remains uncertain. OPN influences bone homeostasis by different mechanisms. This polypeptide chain undergoes extensive post-translational modifications, including glycosylation, phosphorylation and sulfation, and the precise modification pattern depends on the species and tissues in which the protein is synthesized.<sup>7</sup> The functional significance of post-translational modifications in OPN is poorly understood.

Bone remodeling is a regulated process in which removal via osteoclasts is followed by bone formation via osteoblasts.<sup>8</sup> The presence of OPN has traditionally been interpreted as an indicator of bone formation. In bone, OPN is produced by osteoblastic cells at various stages of differentiation,<sup>9</sup> including differentiated osteoblasts, and by osteocytes.<sup>10,11</sup> The protein is primarily made by cells of osteoblastic lineage, and it is also expressed by fibroblastic cells in embryonic stroma<sup>12</sup> and at wound-healing sites.<sup>13</sup> OPN is found *in situ* in osteoblasts and accumulates in mineralized bone matrix during endochondral

and intramembranous ossification.<sup>14</sup> It has also been reported to enhance osteoblastic differentiation and proliferation and increase alkaline phosphatase activity.<sup>15</sup> Increased OPN expression at injury or infection sites likely results from the release of growth factors (e.g., platelet-derived growth factor) or cytokines (e.g., interleukin1) that activate different transcription factors, such as Fos and Jun, which are capable of upregulating OPN transcription.<sup>16</sup>

Hence, besides promoting bone formation, OPN has been implicated in bone resorption.<sup>17</sup> Various mechanisms have been proposed to underlie this biological function. Phosphorylation of OPN appears necessary for the inhibition of biological crystal formation and for the formation of calcium carbonate crystals.<sup>7</sup> OPN is a potent inhibitor of the mineralization process, because it is binding to hydroxyapatite (HA), inhibits the formation of HA crystals<sup>18</sup> and the growth of HA crystals,<sup>19</sup> and promotes the inhibition of bone mineralization.<sup>20</sup> OPN plays an important role in osteoclastogenesis and osteoclast activity. Its expression is upregulated during the maturation of monocytes into macrophages,<sup>21</sup> a process that presumably occurs as circulating monocytes extravasate and migrate through the tissue.<sup>16</sup> Parathyroid hormone-induced RANKL signaling normally augments the number and activation of osteoclasts, but this increase is disrupted in the absence of OPN.<sup>22</sup> The neutralization of OPN suppresses osteoclastogenesis *in vitro*, whereas its addition enhances osteoclastogenesis in OPN<sup>-/-</sup> cells.<sup>23</sup> However, Chellaiah et al. reported an increase in the number of osteoclasts in OPN<sup>-/-</sup> mice as a compensatory mechanism for the decreased activity of OPN<sup>-/-</sup> osteoclasts, because OPN-deficient osteoclasts do not migrate and are unable to resorb bone.<sup>24,25</sup> Hence, bone-resorbing activity could only partially be restored by exogenous OPN,<sup>24</sup> indicating that autocrine OPN is important for osteoclast activity.<sup>26</sup>

Anorganic bovine bone (ABB) is a deproteinized, sterilized bovine cancellous bone comprising calcium-deficient carbonate apatite.<sup>27</sup> ABB is frequently utilized as a bone substitute in maxillary sinus lift procedures when insufficient autogenous cortical bone (ACB) is available for the graft.<sup>28</sup> ABB particles are similar to human cancellous bone in crystalline and morphological structure.<sup>29</sup> They are natural, osteoconductive bone substitutes that promote bone growth in periodontal and maxillofacial osseous defects.

The particles provide a scaffold and a matrix for bone cell migration and are integrated into the natural physiological remodeling process. It has been suggested that deproteinized cancellous bovine bone can induce new bone formation through osteoinductive mechanisms.<sup>30</sup> It has also been reported that the application of ABB in a collagenous matrix induces the formation of membranous and endochondral bone *in vivo* and that ABB exerts high angiogenic activity.<sup>31</sup> In previous studies, however, no OPN was detected in bovine bone slices, and no staining was observed in osteocytes, blood vessels, cement lines or typical sites of OPN expression.<sup>32</sup> In an animal study, Araújo et al. described OPN expression in ABB particles during early healing of the post-extraction socket.<sup>13</sup> Our group described a similar phenomenon in humans during late healing after sinus grafting, observing OPN expression not only in the ABB particles, but also within their canalicular system. These observations differ from previous findings in ultrastructural studies in a rat model that suggested that OPN accumulated at the mineral front and was progressively incorporated deeper into the bone, but by the further deposition of new bone matrix.<sup>33</sup>

The objectives of this study were to examine OPN expression in bone and ABB in maxillary sinus grafts after six months of healing and to study its relationship to morphological and immunohistochemical results and to patient variables and habits.

## Materials & methods

### Study design and subject recruitment

This clinical case series was reviewed and approved by the institutional review board of the University of Granada Faculty of Dentistry (Spain) prior to subject recruitment. The study was conducted according to the principles of the Declaration of Helsinki for experimentation with human subjects.<sup>34</sup>

Totally or partially edentulous patients needing a sinus lift were screened and included in the study if they met the following inclusion criteria: age between 18 and 85 years, Physical Status I or II according to the American Society of Anesthesiologists,<sup>35</sup> absence of uncontrolled systemic disease or a condition known to alter bone metabolism (e.g., osteoporosis or diabetes mellitus), O'Leary plaque score of  $\leq 20\%$ ,<sup>36</sup> and  $\leq 5$  mm of

remaining bone height from measurement on a panoramic radiograph.<sup>37, 38</sup> Exclusion criteria were the following: antibiotic intake in the previous three months, prescription for more than six months of medications known to modify bone metabolism (e.g., bisphosphonates or corticosteroids), pregnancy or intention to become pregnant at the time of the screening, the presence of an untreated chronic sinus condition (e.g., cyst or tumor) or sepsis, a history of cancer or radiation to the oral cavity, complications of these conditions affecting the sinus area, and consumption of > 10 cigarettes/day. Patients smoking up to 10 cigarettes/day<sup>39</sup> and alcohol consumers were included in the study. For the statistical analysis, patients who smoked  $\geq 1$  cigarettes/day were considered smokers and those having  $\geq 1$  alcohol-containing drinks/day (> 10 g of alcohol/day)<sup>40</sup> were considered alcohol consumers. Patients who met the inclusion and exclusion criteria were required to read, understand and sign the informed consent form before being enrolled in the study.

#### Surgical procedures

Patients were asked to take 875/125 mg amoxicillin/clavulanate (or, if allergic to penicillin, 300 mg clindamycin) t.i.d. for ten days, starting two days before the surgery to minimize infection risk. All surgical procedures were performed under local anesthesia (articaine with epinephrine 40/0.01 mg/ml, Sanofi-Aventis Deutschland, Frankfurt/Main, Germany). The procedure proposed by Galindo-Moreno et al.<sup>41</sup> was followed, using a bone scraper (Safescraper, Meta, Reggio Emilia, Italy) to harvest ACB from the lateral wall and expose the Schneiderian membrane. After the membrane lift, sinus cavities were grafted with scraped ACB in combination with ABB particles sized between 250 and 1,000  $\mu\text{m}$  (Geistlich Bio-Oss, Geistlich Pharma, Wolhusen, Switzerland); the ratio of ACB to ABB in the composite graft was 1:1 v/v.<sup>42</sup> A maximum of 5 cc of graft material was used per sinus cavity. After bone grafting, an absorbable collagen membrane (Geistlich Bio-Gide, Geistlich Pharma, Wolhusen, Switzerland) was placed over the lateral aspect of the bony window. Flaps were then carefully approximated and sutured with 3-0 surgical silk (Laboratorio Aragó, Barcelona, Spain) by primary intention.

After a six-month healing period, a trephine (internal and external diameters of 3 mm and

4 mm, respectively) was used to harvest bone core biopsies from the alveolar crest in which implants were prosthetically planned. Implants (OsseoSpeed, Astra Tech, Mölndal, Sweden; Microdent, Microdent Implant System, Barcelona, Spain) were placed in a two-stage approach.

#### Histological study

The trephine biopsies were fixed in 10% buffered formalin for 24 h, decalcified in Decalcifier I (Surgipath Europe, Peterborough, UK), containing formaldehyde (10% w/v), formic acid (8% w/v) and methanol (1% w/v), for 24 h at 37 °C in an oven and embedded in paraffin. Then, 4  $\mu\text{m}$  sections were cut along the central axis of the biopsies and dewaxed and hydrated for staining with hematoxylin–eosin, periodic acid–Schiff, Masson's trichrome and Goldner's trichrome. A millimeter scale in the eyepiece of a BH2 microscope (Olympus Optical, Tokyo, Japan) with a 40 $\times$  objective was used to count osteoblasts, osteoclasts and osteocytes per  $\text{mm}^2$ . Results were expressed in terms of the number of positive cells per  $\text{mm}^2$ .

Bone histomorphometry was performed semi-automatically on Masson's trichrome-stained sections, assessing ten randomized images with a 10 $\times$  objective, using a microscope equipped with a digital camera (DP70, Olympus Optical, Tokyo, Japan) connected to a computer and applying ImageJ software (Version 1.48; developed by the U.S. National Institutes of Health, Bethesda, Md.). Separate quantifications of vital bone, ABB particles and connective tissue were performed, expressing the results as percentages of each compartment.

#### Immunohistochemical analysis

Decalcified and paraffin-embedded sections were dewaxed, hydrated and heat-treated in 1 mM EDTA buffer for antigenic unmasking. Sections were incubated for 60 min at room temperature with pre-diluted OPN polyclonal antibody to identify cellular and interstitial expression and with the following pre-diluted monoclonal antibodies (all from Master Diagnóstica, Granada, Spain): CD34 (clone QBEnd/10) to identify endothelial cells; CD56 (clone 56C04/123A8) to identify osteoblasts; tartrate-resistant acid phosphatase (TRAP; clone 26E5) to identify osteoclasts; CD68 (clone KP1) to identify monocytes and macrophages; and vimentin (clone V9)

Fig. 1

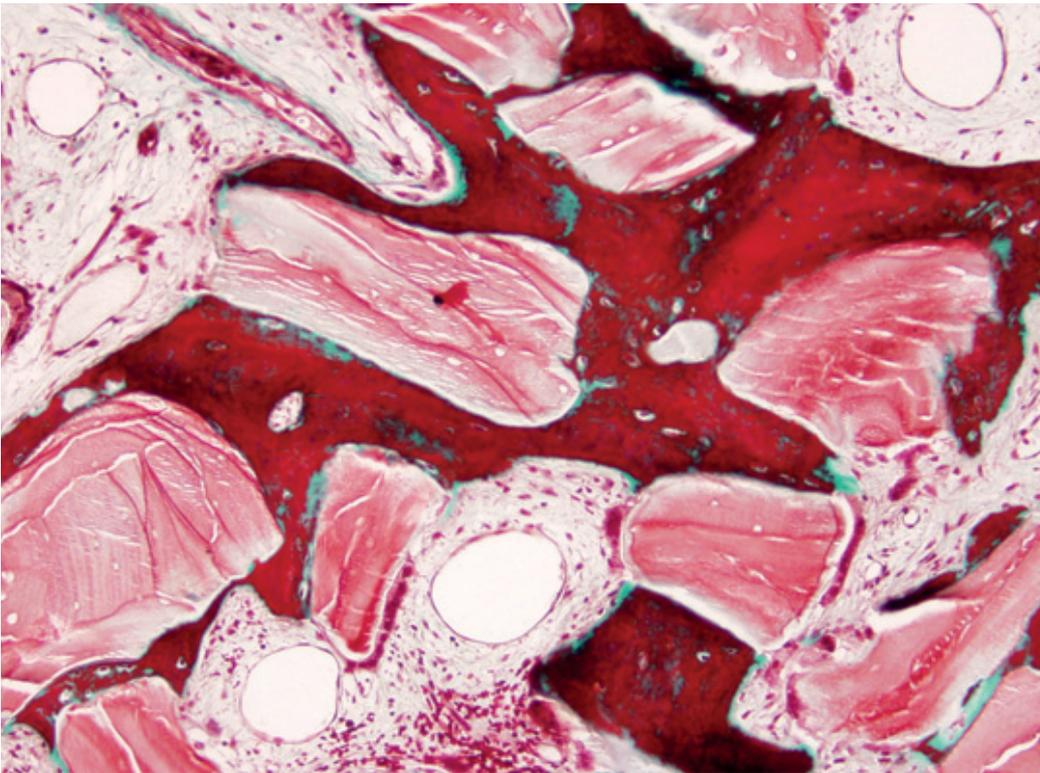


Fig. 1

Trabecular bone formed throughout the ABB particles in a maxillary sinus lift biopsy (Masson's trichrome stain, 10 $\times$ ).

to identify mesenchymal cells (as positive control). The immunohistochemical study was done on an automatic immunostainer (Autostainer 480S, LabVision, Fremont, Calif., U.S.), using the micropolymer-peroxidase-based method followed by development with diaminobenzidine (Ultravision Quanto, Master Diagnóstica, Granada, Spain). A millimeter scale in the eyepiece of a BH2 microscope (Olympus Optical, Tokyo, Japan) with a 40 $\times$  objective was used to count the number of positive cells and vessels per mm<sup>2</sup>.

#### Statistical analysis

The Shapiro–Wilk test was used to assess the normality of variables. After descriptive analysis, the Student's *t*-test or Welch test for unequal variances and the Spearman correlation coefficient ( $\rho$ ) were used to evaluate the significance of differences. Clinical, morphological and morphometric variables were compared between the presence and absence of OPN in cells and ABB particles using the Student's *t*-test. A *p* < 0.05 was considered significant. IBM SPSS Statistics for Windows (Version 20.0; IBM, Armonk, N.Y., U.S.) was used for the data analyses.

## Results

### Histological and histomorphometric results

After six months, a normal woven and lamellar pattern of trabecular bone had formed throughout the graft in all patients who had received ABB plus ACB (1:1) grafts, and biopsies from the augmentation area contained this trabecular bone in different proportions. Image analysis revealed 32.75  $\pm$  14.0% vital bone, 39.49  $\pm$  17.4% connective tissue, and 27.75  $\pm$  21.8% remnant ABB particles (**Fig. 1**). ABB particles were detectable in the trabecular bone in a slightly smaller proportion than in the original graft. In the pristine bone, 48.93  $\pm$  15.4% was vital bone and 51.07  $\pm$  20.8% connective tissue.

### Immunohistochemical results

OPN expression was diffuse in 77.5% (31/40) of ABB samples and focal in 22.5% (9/40); it was diffuse in 80% (8/10) of pristine bone samples and focal in 20% (2/10). The presence of OPN immunostaining on ABB particles was intense in 45% of maxillary sinus lift biopsies, moderate in 27.5%, mild in 10%, and absent in 17.5%; it was distributed within the lacuno-canalicu-

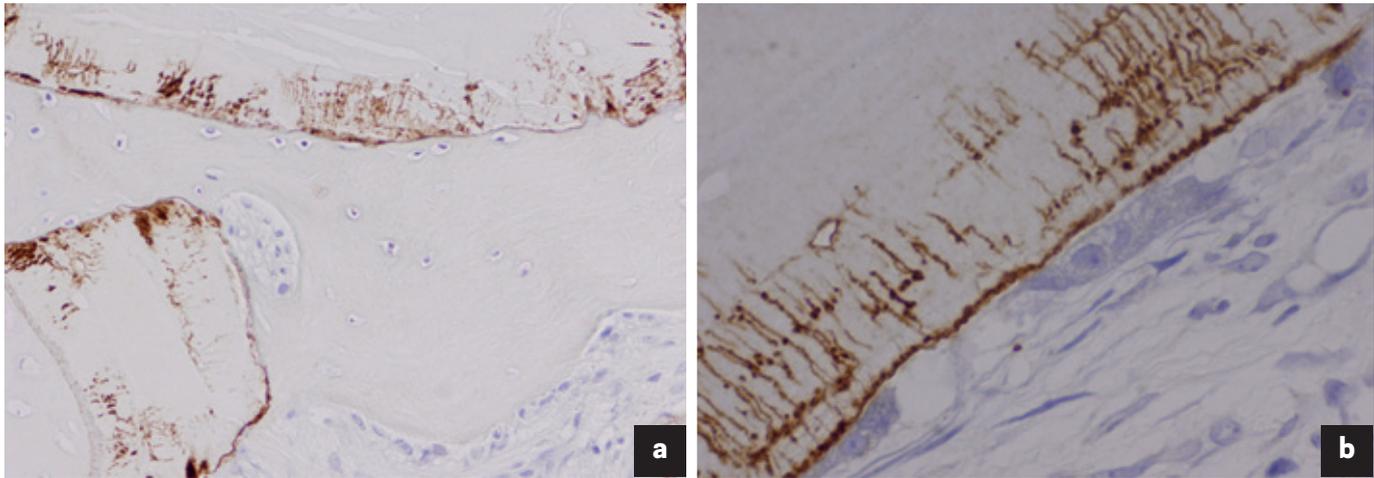


Fig. 2

**Fig. 2**

Immunohistochemical expression of OPN.

**(a)** OPN location on boundary of ABB particles with trabecular bone (micropolymer-peroxidase-based method, 20 $\times$ ).

**(b)** Detail of OPN deposits in lacuno-canalicular network and location on boundary of ABB particles near to osteoclast (micropolymer-peroxidase-based method, 60 $\times$ ).

lar system of ABB particles and on their surface close to osteoclast-like cells (**Fig. 2**).

At six months, OPN expression was principally observed at the interstitial boundary of bone with ABB particles and within lacunae and bone canaliculi, forming a star shape (**Fig. 2**), with no expression in the trabecular bone or interstitium. Cortical OPN expression was directly correlated with the number of osteocytes per mm<sup>2</sup> (rho coefficient = 0.405,  $p = 0.045$ , Spearman test), with OPN expression in cement lines (rho coefficient = 0.757,  $p < 0.001$ , Spearman test) and with OPN expression in osteocytes (rho coefficient = 0.432,  $p = 0.012$ , Spearman test).

A direct correlation was found between OPN expression in ABB particles and in monocytes and macrophages (CD68-positive cells; rho coefficient = 0.583,  $p = 0.009$ , Spearman test). OPN expression in osteocytes was inversely correlated with the number of osteoblasts (CD56-positive cells) per mm<sup>2</sup> (rho coefficient = -0.828,  $p = 0.042$ , Spearman test).

Avascular bed was formed in the nonmineralized tissue by vessels of different calibers in the graft area and by capillary vessels among the adipocytes in the bone marrow area, with a mean in the biopsies of  $86.28 \pm 56.6$  CD34-positive vessels per mm<sup>2</sup>. OPN expression in osteocytes was directly correlated with the number of vessels per mm<sup>2</sup> (rho coefficient = 0.828,  $p = 0.042$ , Spearman test).

TRAP expression was directly correlated with the count per mm<sup>2</sup> of osteoclasts (rho coefficient = 0.532,  $p = 0.015$ , Spearman test), monocytes and macrophages (rho coefficient = 0.622,  $p = 0.008$ , Spearman test), and osteoblasts (rho coefficient = 0.391,  $p = 0.048$ , Spearman test). TRAP expression was correlated with the local and diffuse expression of OPN (rho coefficient = 0.439,  $p = 0.022$ , Spearman test; **Fig. 3**).

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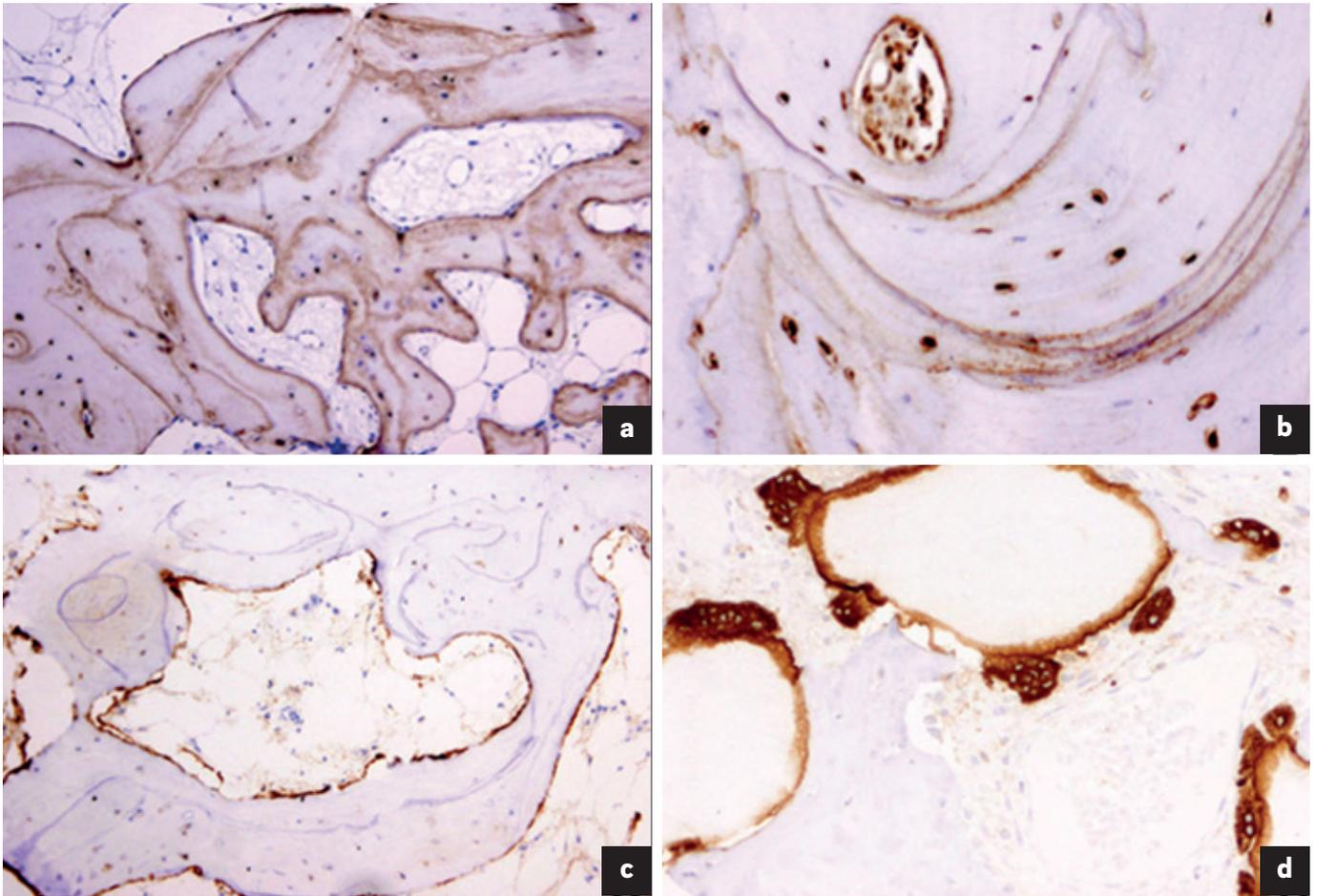
## Discussion

In this study of bone xenografts in a clinical series of maxillary sinus lift, immunohistochemical OPN expression was detected not only in osteocytes and on ABB particles, but also within the lacuno-canalicular system of ABB particles and close to osteoclast-like cells on their surface.

Bone formation or resorption requires adhesion molecules (arginine-glycine-asparagine sequences), such as fibronectin, fibrinogen, vitronectin, Type I collagen, OPN or bone sialoprotein, to attach osteoblasts or osteoclasts to surfaces for remodeling.<sup>43</sup> Because ABB particles are free of proteins,<sup>44</sup> protein expression on the particles must derive from proteins absorbed from the environment. The above chemotactic factors may have stimulated and directed the migration of cells to the foreign material.<sup>13</sup>

Microchannel pores (< 10  $\mu\text{m}$ ) of ABB may be relevant for osteogenic cell attachment, migration, proliferation and differentiation.<sup>45</sup> At the same time, the inner surface of ABB particles becomes considerably enlarged, favoring the formation of new vessels and therefore the inward growth of new bone within the particles.<sup>31</sup> A greater microporosity also expands

Fig. 3

**Fig. 3**

**(a)** Immunohistochemical expression of OPN in osteocytes and cement lines in pristine bone (micropolymer-peroxidase-based method, 10 $\times$ ).

**(b)** Detail of OPN expression in osteocytes (micropolymer-peroxidase-based method, 20 $\times$ ).

**(c)** Immunohistochemical expression of CD56 in osteoblasts around trabecular bone (micropolymer-peroxidase-based method, 10 $\times$ ).

**(d)** Immunohistochemical expression of TRAP in osteoclasts around ABB particles (micropolymer-peroxidase-based method, 40 $\times$ ).

the scaffold surface and may enhance cytokine adsorption.<sup>46</sup> The interconnectivity of the pores in ABB particles and their hydrophilic properties explain the presence of OPN within the lacuno-canalicular system. This explanation is supported by the distribution of OPN found in our samples from the surface to the core of the particles, with the lacuno-canalicular system of the remnant ABB particles clearly depicted by the staining. Although the biological relevance of this finding remains unclear, it may be related to the cellular recolonization and revascularization of ABB that has been observed after six months of healing.<sup>31</sup> In this previous study, a direct correlation was found between OPN expression in osteocytes and CD34-positive endothelial cells (rho coefficient = 0.828,  $p = 0.042$ , Spearman test). As already noted, one of the functions of OPN is related to angiogenesis,<sup>5</sup> which is impaired in OPN-deficient mice.<sup>47</sup> Images of ABB particles after six months of graft maturation were compatible with neovascularization and central re-

sorption, implying the integration of this biomaterial within the functional and biomechanical system of the neoformed bone.

Whereas most noncollagenous proteins are more or less homogeneously dispersed throughout bone, ultrastructural immunocytochemical studies have consistently found OPN to be predominantly distributed at cement lines in remodeling bone and at laminae limitans.<sup>48,49</sup> These sites represent matrix–matrix and cell–matrix boundaries, respectively, and are therefore important in the bone formation process.<sup>6</sup> Cement lines demarcate the boundary between older and newer bone and characteristically have a high OPN content. The origin of OPN in the cement line has been controversial,<sup>32,50</sup> and osteoclasts and osteoblasts may both be involved. OPN may initially play a role in osteoblast adhesion or early calcification events in the cement layer.<sup>51</sup> Subsequently, the more diffuse distribution of OPN throughout the bone matrix may influence osteoclast activity during resorption and the transformation of woven and lamellar bone.<sup>6</sup> Our findings support this proposi-

tion, given that OPN expression in the cement lines may indicate bone formation or destruction as a function of the cells attached to the particle surface. However, this dual behavior of OPN remains unclear.

OPN can be expressed in numerous tissues<sup>52</sup> and by multiple cell types,<sup>14</sup> including osteoblasts and osteoclasts.<sup>53</sup> Kunii et al. confirmed the expression of OPN mRNA and protein in human osteocytes<sup>32</sup> using different antibodies.<sup>52</sup> Osteocytes have multiple functions and regulate different processes in bone homeostasis.<sup>54</sup> After six months of healing, osteocytes present in the new vital bone in our biopsies consistently expressed OPN. OPN expression in osteocytes was inversely correlated with the count of osteoblasts (CD56-positive cells) per mm<sup>2</sup> (rho coefficient = -0.828,  $p = 0.042$ , Spearman test). Araújo et al. detected OPN expression on ABB particles in dogs during the early stages of healing.<sup>13</sup> According to their study, after the migration of polymorphonuclear cells to the ABB surface, these cells are replaced by TRAP-positive cells (osteoclasts), which will remove material from the surface of the xenograft.<sup>13</sup> Subsequently, osteoblasts will attach to the remodeled surface and bone apposition will begin. However, OPN behavior is likely different during the late stage of healing. In the present samples, osteoblasts were rarely observed in the ABB particles, and a significant inverse relationship was found between OPN expression and CD56 expression (rho coefficient = -0.828,  $p = 0.04$ , Spearman test). This higher expression with fewer osteoblasts and the positive correlation between OPN and CD68 expression (rho coefficient = 0.583,  $p = 0.009$ ; rho coefficient = 0.938,  $p = 0.006$ ; Spearman test, respectively) indicate that OPN expression on ABB particles promotes resorption at six months of healing. In support of this hypothesis, it is known that OPN can both activate osteoclasts for bone matrix resorption and induce their migration in an  $\alpha_v\beta_3$  integrin-dependent manner.<sup>55,56</sup> Nakamura et al. demonstrated that  $\alpha_v\beta_3$  integrin plays a key role in osteoclast migration, which is essential for efficient osteoclastic bone resorption.<sup>57</sup> Perrotti et al. reported that multinucleated cells generated on ABB were positive for the  $\alpha_v\beta_3$  subunit of vitronectin,<sup>58</sup> the main integrin-mediating osteoclast cell attachment to the bone matrix;<sup>59</sup> hence, this mechanism may be functional on ABB particles. OPN is a ligand for the  $\alpha_v\beta_3$  integrin through an RGD sequence.<sup>60</sup> The osteoclast cell membrane must be sealed to the substrate by means of cell surface

receptors and proteins of the integrin family before beginning its proteolytic activity. The binding of OPN to the  $\alpha_v\beta_3$  integrin in the sealing zone or podosomes appears essential to the reorganization of the actin cytoskeleton for osteoclast motility.<sup>61</sup> Additionally, osteoclast adhesion and osteoclast migration are mediated by phosphorylated OPN,<sup>62</sup> and this biological event is regulated by endogenous TRAP.<sup>63</sup> Interestingly, TRAP and OPN expressions showed similar patterns on ABB particle surfaces at six months of healing in our samples, indicating the close relationship between OPN expression and the osteoclastic resorption of ABB. Questions have been raised about this resorption of the particles,<sup>64,13</sup> but this proposition is supported by our histological findings on these particles of bone-remodeling units with multinucleated cells in different stages of differentiation (CD68 positive and TRAP positive) that promote these phenomena. In our view, these results may in part explain OPN-mediated ABB resorption during the late stage of graft healing.

Our findings support the proposal that osteoclasts are the source of OPN in bone cement lines during remodeling.<sup>32</sup> The detection of OPN expression within lacunae represents clear evidence of the secretion of OPN by osteoclasts, because exogenously added OPN has no access to these sites.<sup>23</sup>

## Conclusion

Immunohistochemical expression of OPN is related to bone remodeling and maturation changes in maxillary sinus lift with ABB xenograft.

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## Competing interests

The authors declare that they have no competing interests. This investigation was supported in part by Research Groups #CTS-138 and CTS-583 (Regional Government of Andalusia, Spain).

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