ORIGINAL ARTICLE

RUNX2, BMP4 AND SPARC EXPRESSION IN BONE REGENERATION FOLLOWING BONE SUBSTITUTES BASED SINUS AUGMENTATION HAVE IMPACT ON DENTAL IMPLANTS FAILURE

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ABSTRACT

RUNX2, BMPs and SPARC evaluation on oral tissues was reported in experimental studies on gingival cells with osteogenic potential important for guided bone regeneration. Data regarding these markers, in tissues, other than bone, is extremely limited, in literature, especially regarding their involvement in periodontitis pathology and dental implants success rate. The aim of this pilot study is to identify an immunophenotype in order to prove the correlation between three markers (RUNX2, BMP4, SPARC) and the implant survival rate after sinus lift augmentation by using as biomaterial spongy and cortical bone of equine origin, in patients with treated periodontal diseases. Ten patients were recruited. Only 2 patients, based on inclusion/exclusion criteria completed the study. Soft tisues and bone chips samples were collected at three different moments: before initial periodontal therapy (T^2), during the sinus augmentation surgery prior the implant (T^1) and after removal of unsuccessful implants (T^2). The samples were immunohistochemically evaluated for RUNX2, BMP4 and SPARC. RUNX2low or absent/SPARChigh/BMP4high found inside epithelium and adjacent connective tissue, caracterized the periodontitis and failured implants. RUNX2high/SPARClow/BMP4low was specific for successful implants. Conclusion. RUNX2/SPARC/BMP4 is a proper panel marker to evaluate the quality and the regenerative potential of the insertion site before implant placement.

Keywords: RUNX2, BMP4, SPARC, immunophenotype, periodontitis, bone regeneration, maxillary sinus augmentation, dental implants.

INTRODUCTION

It is well known that RUNX2, BMPs and SPARC are markers that evaluate osteoprogenitor and osteoblast cell type in bone regeneration process, characteristic of young bone cells [1]. Only several experimental studies reported the potential of gingival cells with osteogenic differentiation at the gingival tissue and periodontal ligament level. This process is essential in bone regeneration [2,3].

RUNX2 marker has been studied most extensively followed by BMPs and to a lesser extent SPARC. Bone morphogenic proteins (BMPs) are growth factors for head and neck tissues development, generally, including the structures of oral cavity and particularly teeth and periodontal structures. Both, in healthy patients or patients with a history of periodontitis most extensively studied BMP markers are BMP2, 7 and 9. If BMP2 is intensively studied, BMP4 with a high structural similarity has long been neglected in the study of periodontal lesions. There are currently only 25 studies evaluating BMPs in periodontitis, especially BMP2 and BMP9. BMP4 is poorly studied and only indirectly mentioned in the evaluation of chronic periodontitis [4, 5] most often in relation to their role on gingival fibroblasts [6]. Secreted by osteoblasts, the osteonectin or SPARC is involved in bone mineralization, not specific to bone cell, but expressed in periodontal ligament cells in gingival fibroblasts and linked to collagen fibers. In pathological conditions SPARC expression has been reported in several malignant tumors [7, 8, 9]. It has also been evaluated in periodontitis, but not accepted yet, as a salivary marker for the assessment of bone destruction. Among other functions, SPARC plays a major role in the wound healing response to injury and tissue remodeling due to macrophages [10].

Endothelial cells, under inflammatory conditions, express SPARC with a direct role in their activation and proliferation [11, 12]. Recently SPARC is recognized as a fibrosis-favoring factor, certified at the liver level [13, 14] and extensively discussed in periodontal inflammatory lesions, especially in the periodontal ligament [15]. This imunophenotype RUNX2, BMP4 and SPARC has also been associated with bone matrix synthesis and

mineralization [16].

The correlation between these markers and periodontal pathology is poorly documented and the study of these three markers in chronic periodontitis, in other tissues than the bone, is extremely limited.

The above mentioned reasons were the basis for this pilot study, evaluating the influence of RUNX2, BMP4, SPARC in 2 stage lateral windows sinus augmentation and 9 months delayed implant placement success rate in patients with history of severe periodontitis.

MATERIAL AND METHODS

The pilot study was approved by the Institutional Ethical Committee of „Victor Babes" University, Timisoara, and the Review Board of the Government Dental College and Research Institute and conducted in in accordance with the declaration of Helsinki. All participants were verbally informed regarding sampling procedure, timepoints and conditions to be met. Written informed consent was collected from all participants.

Ten patients, 6 men and 4 women, age range: 25 and 67 years, mean age: 45,7 with generalized advanced periodontitis, after periodontal initial therapy, underwent minimum one sinus floor augmentation following a 2nd-stage surgery with implant placement after 9 month. The inclusion criteria were: 1) no systemic diseases that could influence the outcome of the surgery; 2) generalized severe periodontitis; 3) a moderately atrophic lateral maxilla certified by Cone Beam Computed Tomography (CBCT); 4) Hounsfield unit (HU) score recorded on CBCT prior the implant placement, 9 month after sinus augmentation ≤ 199 HU. Exclusion criteria were: 1) patients with systemic diseases known to affect the outcome of surgical therapy; 2) immune-compromised individuals; 3) pregnant or lactating females, 4) diabetes, 5) tobacco smokers or tobacco use in any form, 6) non compliant patients, 7) prolonged antibiotic treatment or anti-inflammatory treatment within 6 months prior the surgery, 8) hematologic disorders or insufficient platelet count (< 200.000/mm3). Bone density of the trabecular posterior maxilla in heathy subjects was reported, by previous studies, to have a mean value of 199 HU (17).

For sinus floor augmentation there were used granules of spongy and cortical bone of equine origin, fully enzyme deantigenised (BIO-GEN®, Bioteck, Vincenza, Italy) resuspended in Hyaluronic acid (H) (Hyadent BG, Bio Science, Dummer, Germany) and combined with collagen membrane.

Out of the ten patients that underwent sinus augmentation, eight patients have recorded an HU score of 350-450 HU and based on inclusion criteria were excluded. The two remaining patients with bilateral sinus augmented, recorded scores lower than the mean of 199 HU and completed the pilot study.

The implants were inserted and four months later, the implants with periimplantitis, granulation tissues and osteolysis were considered as to be failed. For these patients we harvested biopsied as it follows: T□ - before parodontal therapy from dental alveolar after removal of the affected teeth; T^1 - from the anterior wall of the sinus during sinus lifting procedure before bone augmentation; T2 - from dental alveolar structures after removal of unsuccesfull implants.

The harvested biopsies were morphologically analyzed and subsequently quantified for the immunohistochemical expression of the main factors involved in periodontal tissue regeneration such as Runt related-RUNX2 transcription factor, the osteonectin (SPARC) and the morphogenetic bone protein 4 (BMP4).

Immunohistochemistry

Immunohistochemistry was used to quantify the presence of osteoblastic commitment of mesenchymal cells osteoblastic differentiation and bone formation. Three different antibodies were selected. We used rabbit polyclonal anti RUNX2 (Runt-related transcription factor 2) antibody (Santa Cruz Biotechnology dilution 1:100) a key transcription factor for osteoblast differentiation; rabbit polyclonal anti SPARC (osteonectin) antibody (Abcam dilution 1:100) a bone glycoprotein secreted by osteoblasts; and rabbit polyclonal anti BMP4 (bone morphogenic protein) antibody (Novus Biological dilution 1:500) which appear during bone repair. Incubation with primary antibody for 30 min at room temperature was followed by the use of the visualization system Novolink Max Polymer/DAB. All immunohistochemistry steps were fully controlled using Max Bond Autostainer (Leica Microsystems, Medist Life Sciences, Bucharest Romania).

Microscopic evaluation

Microscopic evaluation of the specimens was performed using an Axio Zoom 2 Observer Microscope (Zeiss, Germany) and pictures from different moments of the experiment were obtained and processed with ZEN software (version 2, Zeiss, Germany).

RESULTS

The HU values recorded for the first patient were lower (100-200) only on the right side compared to the left side where the values were whithin the range and in some areas The second patient had low values of bone density in both sides where augmentation was performed.

Clinico-morphological, immunohistochemical and imagistic findings. Two patients were chosen, considered to be significant from the point of view of the achievement of clinical/morphological/imagistic and immunohistochemical correlations and who have required multiple dental extractions, sinus lifting and dental implants and from whom tissue samples were taken in different stages of the applied therapy.

First patient: OG, 49 years old, paraclinical data within normal range except for total lipids and cholesterol required sinus elevation (Fig. 1). The radiologic image

correlated with the morphological one, is presented below:

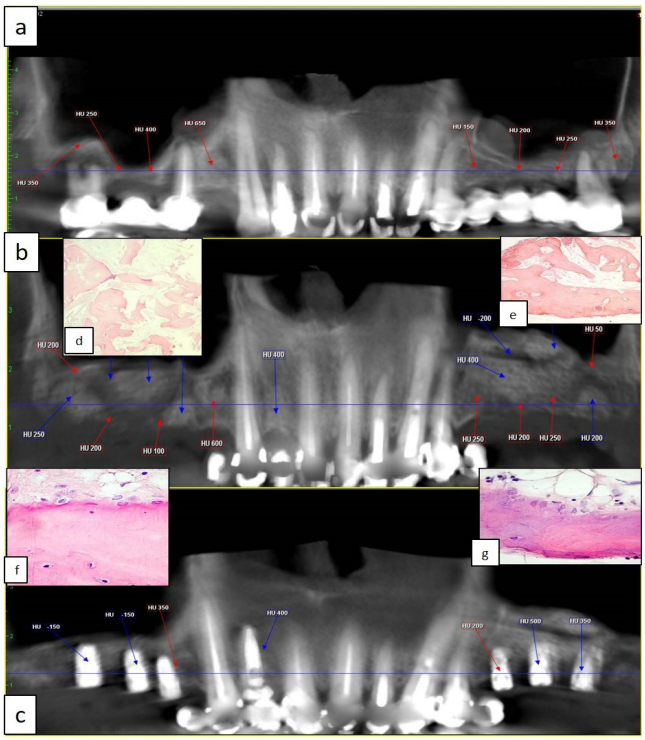

Figure 1.

. Morphologic and imagistic evaluation of the patient related to osteodensitometry. Osteodensitometric evaluation before augmentation, (a) after augmentation (b) and postimplant (c). Note more compact appearance of one on the right compared with the left part(b) certified by the microscopic images (d, e). No significant radiological differences have been found postimplant (c) but microscopy proved the low density of osteoblast on the left side (f) compared with the right side (g).

For this patient, the radiological osteo-densitometric data was not significantly different between the left and right maxillary alveolar bone before augmentation (Fig. 1a). The radiological evaluation of the bone, post augmentation included the assessment of osteo-densitometry favorable for achieving an optimal dental implant but also an evaluation of the ratio between the alveolar bone itself and the used biomaterial (Fig. 1b). Even though the same material was used, the radiological data showed and supported the different reactivity of the alveolar bone for the two parts of the maxilla. Radiologically speaking, the post-augmentation alveolar bone on the left side showed the presence of high bone density areas that alternated with relatively frequent areas of low bone density areas frequently interposed across high density areas suggesting either an incomplete apposition of the used biomaterial or a faulty alveolar bone regeneration (Fig. 1d). Conversely, for the right side of the maxillary the structure of the alveolar bone after bone augmentation was much more homogeneous with predominantly high bone density areas the low density bone areas being very rare (Fig. 1e). The microscopic analysis of soft and bone tissues harvested pre and post-bone augmentation sustained the presence of these radiological and osteo-densitometric differences, which were related to the histological structure of the bone as well as to cellular reactivity cell type and the presence of immunohistochemical markers of evaluation of their osteoblastic character.

However, the morphological data reported significant differences between the two parts of the maxillary regarding the histological structure of the alveolar bone but also the bone cellularity and the state of the tissue at the spaces between the bone lamellae level. On the right side, the bone lamellae were relatively complete the bone trabeculae being interconnected and separated by connective tissue without inflammatory infiltration (Fig. 1g). In contrast, for the left side even if osteo-densitometry was relatively close to the previous one the bone lamellae obtained on bone biopsy were damaged showing an irregular contour with lack of lamellae interconnection or with broken bone lamellae with interlamellar connective tissue but also fibrous connective tissue with discrete inflammatory infiltration (Fig. 3f). Another morphological aspect correlated with this different bone density was that the density and the osteoblasts at the surface of the bone lamella were different between the two sides of the maxilla. If on the right side the number of active osteoblasts was significantly higher and the bone structure showed relatively frequent osteoid areas (Fig. 1g, which suggests an increased ability of the bone to regenerate), the left side was characterized by rare osteoblasts and the predominance of mature bone tissue without evident osteoid deposition.

At the mandibular level osteodensitometry presented optimal values for inserting the implants for which the implant procedure was performed. The patient progressed favorably post implant.

The post implant evolution was in accordance with the clinical, microscopic and immunohistochemical data. The dental implant was tolerated on the right side of the maxillary while the left side implant, in a morphologically unstable alveolar bone did not have a favorable evolution, the failure of bone augmentation therapy being registered followed by the dental implant.

The second patient also required bone augmentation that preceded the implant surgery. The same type of augmentation material, Bio Oss associated to the collagen membrane was also used for this. Also, histopathologic and immunohistochemical soft tissues evaluation and bone fragments completed the radiological and osteo-densitometric panel previously evaluated.

The variability of osteo-densitometric results was significantly increased in this patient versus the previous one. Bone density was reduced prior to bone augmentation procedures (Fig. 2a) and did not significantly increase after guided bone regeneration technique (Fig. 2b).

The initial diagnosis of this patient was severe chronic generalized periodontitis, microscopically

certified by the presence of a rich inflammatory infiltrate in the own lamina of the gingival mucosa but also abundantly intraepithelial with the extensive destruction of the gingival epithelium. The microscopic evaluation of the alveolar bone noted unlike the previous case the presence of the abundant inflammatory infiltrate in the tissue between the bone lamellae aspect that has been observed both in the tissues harvested before the bone augmentation and post augmentation. Also, the inflammatory infiltrate persisted in the gingival mucosa after the augmentation procedure (Fig. 2e) even the presurgical therapy was performed and there was no sign of visible inflammation at the moment of surgery. Microscopically it was observed the bone lamellae partially degraded (Fig. 2c), extended areas of fibrosis being detected between bone lamellae mixed with augmentation biomaterial (Fig. 2d).

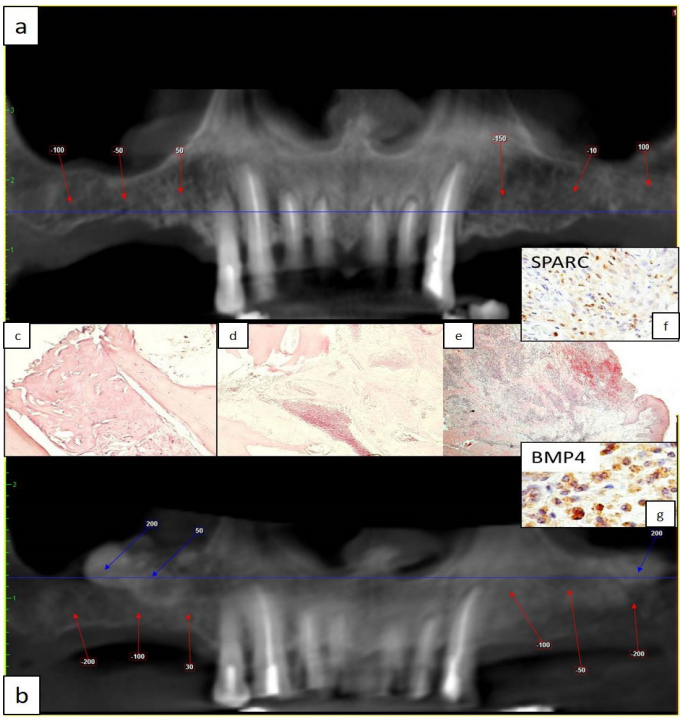

Figure 2.

Morphological osteo-densitometric and immunohistochemical data relevant for the implant failure.

Expression and significance of RUNX2 in preimplantation lesions and in graft rejection after application of guided tissue regeneration with BioOss and collagen membrane.

RUNX2 was present both in the bone and soft periodontal tissues, its expression being dependent on the time and the status of harvested tissue.

In gingival mucosa fragments harvested in the initial stage (T0) RUNX2 expression was weak being restricted to the epithelial cells of the basal layer of the gingival epithelium as well as to the endothelial cells of the intraepithelial vessels frequently found in the gingival epithelium modified by periodontal disease.

In the gingival mucosa harvested six months after the initiation of mandibular therapy (T1) no RUNX2 positive cells were observed neither in the gingival epithelium, nor in the subepithelial connective tissue which showed a discrete inflammatory infiltration.

On biopsies harvested in T2 stage, RUNX2 presented a heterogeneity expression at the anterior sinus wall level (right and left) at the bone level RUNX2 positive osteoblasts being present at the right maxillary sinus level (Fig. 3a) and absent in the left maxillary sinus level prior to the application of sinus lifting techniques (Fig. 3b) inside the tissues harvested from the first patient. Furthermore the dental implants were successfully performed on the right maxillary sinus and failed on the left one.

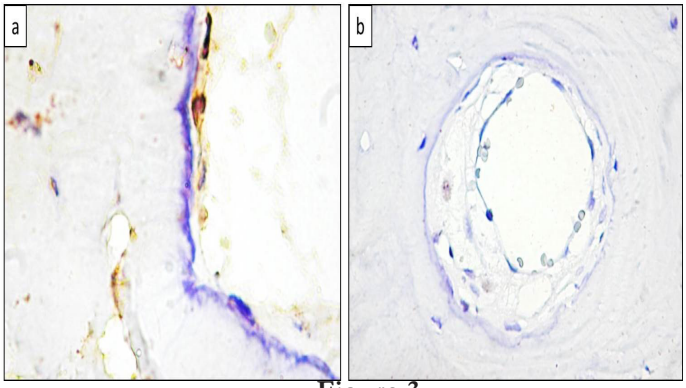

Figure 3.

RUNX2 positive osteoblasts at the bone level in the anterior wall of the right maxillary sinus (a) and their absence in the harvested sample from the left maxillary sinus (b).

On the mandible the RUNX2 expression was different compared to the one from the maxillary level. Cells density at the gingival mucosa epithelium level was significantly increased and RUNX2 was also observed in remaining osteoblasts at the surface of the alveolar bone. The inflammatory cells in the gingival connective tissue as well as the connective cells themselves were negative for RUNX2 in the mandibular gingival mucosa.

SPARC's role in changes occurred in periodontal tissues pre and post implant.

Just like RUNX2, SPARC was evaluated in the periodontal tissues and bone before and after the sinus elevation techniques.

SPARC expression was directly proportional with the degree of inflammation being expressed in gingival epithelial cells, endothelial cells and inflammatory infiltrate of the connective tissue of the gingival mucosa (Fig. 2f). Thus, in initial inflammatory lesions we have observed SPARC expression only isolated in endothelial cells the other cell types being negative. In contrast, in severe periodontal lesions, characterized by a rich inflammatory infiltrate (T0) the SPARC expression was extended and heterogeneous. In gingival epithelium, basal cells showed an increased reactivity and density for SPARC in severe periodontal lesions. The nuclear expression of SPARC has characterized the basal and parabasal layer cells with moderate and high intensity. In the superficial layers SPARC expression intensity was directly proportional to the degree of destruction of the gingival epithelium, being most pronounced in the areas with disorganized epithelium and invaded by intraepithelial capillary blood vessels. Also, the endothelial cells of these vessels have strongly expressed SPARC with nuclear localization as well.

Inside bone, SPARC was expressed in osteocytes most

likely in reactive osteocytes inside the bone tissue of anterior maxillary sinus wall fragments harvested prior to bone atrophy treatment. We did not notice positive osteoblasts for SPARC in the maxillary bone (Fig. 4).

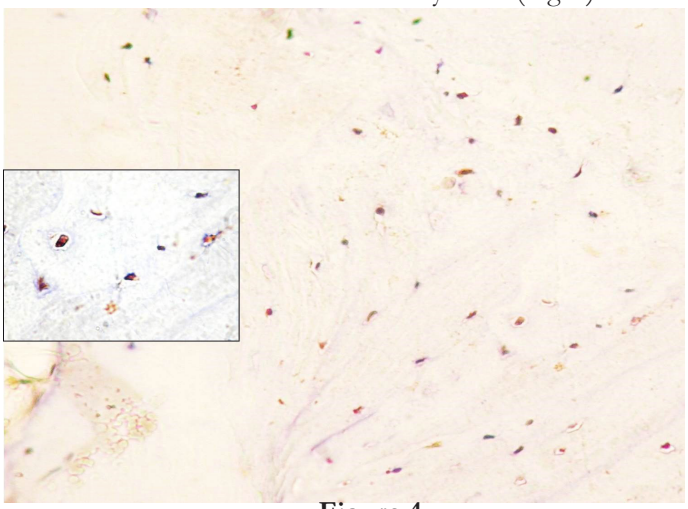

Figure 4.

SPARC positive osteocytes within the bone harvested from the level of the anterior wall of the maxillary bone prior to performing the sinus lifting procedures. BMP4 as stromal and guided tissue regeneration factor.

In periodontal lesions (T0), BMP4 expression was present in gingiva connective tissue and intraepithelial inflammatory infiltrate cells compared to post-surgery collected tissue where it was restricted only at the connective tissue level. Epithelial cells as well as endothelial cells were BMP4 negative, only intraepithelial BMP4 expressing lymphocytes being observed. In the gingival covering mucosa above the implants, BMP4 expression was extremely reduced being present only inside connective tissue macrophages.

Based on the immunohistochemical evaluation of these 3 markers we observed that RUNX2high/SPARClow/BMP4low immunophenotype was specific for successful implants because of high amount of RUNX2 positive osteoblasts found on bone lamellae surfaces and limited expression of inflammatory markers SPARC and BMP4 in cadjacent connective tissue. The implants failure found in the second case was characterized by RUNX2low or absent/SPARChigh/BMP4high immunophenotype, SPARC and BMP4 being highly expressed inside epithelium and adjacent connective tissue, with a similar expression as it has been observed from tissues with periodontitis .

DISCUSSION

The effects of bone regeneration therapy depend mostly on the correct radiological and osteo-densitometric evaluation of bone defects and on the clinical skills and experience of the operator. Despite the fact that advancements in implant dentistry have led to increased predictability of such therapies, implants that seem to have a good prognosis after bone regeneration therapy, sometimes fail without any clinical or radiological cause.

Two of the most common causes of dental implant failure are: mechanical overload and peri-implant inflammation [15, 16]. A higher prevalence of peri-implantitis has been identified for patients with history of periodontal disease, poor plaque control and lack of regular maintenance care after implant therapy. Evidence suggest that progressive crestal bone loss around implants, in the absence of clinical signs of soft tissue inflammation, is a rare event [17].

Regular plaque removal is an important strategy in the prevention and management of peri-implant mucositis, deterring it from progressing to peri-implantitis. Mechanical plaque control should be considered the standard of care for the maintenance of periodontal health around the dental implant and it should be performed either by the patient or the oral healthcare professional [18].

Similar to periodontitis lesions, bacterial plaque accumulation around dental implants causes molecular changes both in the gingival tissues as well as at the level of the augmented bone surrounding the implant. Most available data on this topic has been collected from experiments [19, 20, 21] or, to a smaller extent, from the histo-pathological study of human peri-implantitis lesions [22, 23, 24].

Due to the fact that the prevalence of peri-implant lesions is relatively high new therapeutic agents with an anti-inflammatory role or role of stimulation of osteoblastic differentiation is imperiously needed.

A preliminary molecular evaluation of peri-implant lesions was published by Schminke et al., referring to the post-implant alveolar bone pathology following the occurrence of peri-implant inflammatory lesions [25]. Thus, the authors observed a decrease of the expression of the bone matrix proteins correlated with the decrease of the RUNX2 expression in osteoblasts of the alveolar bone affected by peri-implant lesions.

The other two markers chosen for our study, SPARC and BMP4 are recognized as having a major role in bone mineralization as well as in the development of oral cavity tissue with direct implications in osteogenesis. SPARC tends to become a salivary marker of bone destruction, its values being inversely proportional to the degree of bone destruction. Like RUNX2, data regarding the variability of BMP4 and SPARC expression are reported mainly in relation to bone tissue and more rarely with their expression in gingival soft tissues. For these reasons, we have considered it appropriate to study their expression both in bone tissue and soft periodontal tissues, which could influence the condition of the pre- and post-implant alveolar bone. The SPARC expression in our study was directly proportional to the degree of inflammation in soft tissues and restricted at the level of active osteocytes in the case of the tissues for which no inflammation was detected and where the dental implant subsequently favorably evolved. These results suggest the dual role of SPARC in periodontal tissues and in the regenerated alveolar bone. The dual role of SPARC has been mentioned in the literature for other pathologies, such as bleomycin-induced pulmonary fibrosis [26]. Thus, the SPARC function is dependent on the cell type that secretes this protein, the SPARC secreted by leukocytes originating in the haematogenous marrow limits the fibrosis and reduces the degree of inflammation. The increased expression in periodontal tissues with pre- and post-implant severe

inflammation can be explained as part of a regenerative, healing mechanism for periodontal lesions knowing that SPARC is synthesized by macrophages in extensive healing and regeneration processes. At the same time, SPARC is well known as being a protein with anti-adhesive properties, being recognized and studied more strongly as a promoter of tumor cell migration. The interrelation of the SPARC expression in connective tissues in relation to the bone tissue represents an intensely studied field in the formation of bone metastases [27]. By analogy with the previously reported SPARC anti-adhesion effects, SPARC overexpression in soft periodontal tissues epithelial tissue and before and after guided tissue regeneration techniques can be considered a negative prognostic factor for the therapeutic success of the integration of the BioOss graft and the collagen membrane by lowering its adhesiveness to the host's alveolar bone. Also, the persistence of the positive SPARC inflammatory infiltrate at the time of the insertion of the implant may affect the contact between the implant itself and the host tissues [28, 29, 30].

BMP4 represents the paradox of research on periodontal lesions and alveolar bone regeneration in the sense that despite being well-known as a "key" factor in dental morphogenesis and embryology and tissues at the oral cavity, it is poorly studied in periodontal lesions, peri-implant lesions and alveolar bone regeneration after applied guided tissue regeneration techniques. rhBMPs are used in oral surgery as adjuvant therapy for regenerative purposes [28, 29, 30, 31].

Like SPARC, BMP4 plays a dual role in the pathogenesis of periodontal lesions and in the regeneration of the alveolar bone. As in the inflammatory degenerative lesions in rheumatoid arthritis, the alveolar bone from the severe periodontal lesions appears to be the "target and victim" of the inflammatory cells. BMP4 is a mandatory factor of differentiation of osteoblasts from mesenchymal cells. BMP4 is abundant in the connective tissue of the head and neck and it actively participates in the morphogenesis of the oral cavity. Its persistence determines the maintenance of mesenchymal cells in the immature, undifferentiated stage [32, 33]. This aspect has been experimentally certified by testing the mesenchymal cells of the periodontal ligament in culture treated with BMP4 and studied in terms of expression of markers that certify undifferentiated cell appearance (OCT3 / 4, Sox2 and Myc). The addition of BMP4 led to the preservation of the non-differentiated immaturity phenotype at the 7th passage compared to the lack of BMP4, which kept the immature phenotype only up to the third passage [34]. These evidences indirectly explain the absence of BMP4 expression in osteoblast like cells in peri-implant inflammatory lesions and in the tissues harvested after bone augmentation with biomaterial observed in our study, suggesting an incomplete osteoblastic differentiation capacity followed by a defective osteogenesis with the formation of an alveolar bone of improper quality for the achievement of the dental implant.

The interrelation between BMP4, its expression in the inflammatory infiltrate and the defective osteoinductive process observed in cases of persistent inflammatory lesions is little, if not at all studied in the sphere of periodontal lesions before or after the dental implant or bone augmentation. At the level of gingival inflammatory infiltrate, at least two cell populations secret BMP4: plasma and lymphoid cells. Correlated with the data described above, the over expression of BMP4 in gingival connective tissue with abundant inflammatory infiltrate may have a negative role in alveolar bone regeneration on one hand by restricting differentiation of multi potent stem cells towards the osteoblastic line and on the other hand by a mechanism similar to that described for the progressive ossifying fibroid disorder. In this type of disorder, BMP4 secreted by a subpopulation of abnormal lymphocytes leads to poor skeletal ossification and initiation of aberrant ossification phenomena in soft muscular and connective tissue.

The agglomeration of BMP4-positive cells immediately under the epithelium observed in our study supports the involvement of this protein in the gingival epithelium regeneration under the influence of underlying connective tissue and BMP4, most likely by a mechanism similar to that known from the embryonic development and differentiation of primitive oral cavity tissues.

CONCLUSIONS

This study focused on the characterization of RUNX2 / SPARC / BMP4 expression before and after the bone augmentation and the dental implant, given the fact that this panel characterizes both the osteoblast capacity of the osteoforming cells and the inflammatory aspects of the peri-implant lesions. RUNX2 expression was different in between two patients, being absent in the patient who experienced an abundant and persistent inflammatory infiltrate even after the bone augmentation procedures were performed. A particular aspect of the present study may be the fact that at in areas where the RUNX2 expression was negative or reduced in the tissue harvested after bone augmentation prior the implant insertion, the dental implant was followed by an unfavorable evolution. Our study noted the SPARC overexpression in inflammatory infiltrating cells with the particularity that the macrophages expressed more intensely SPARC than other inflammatory infiltrate cells. BMP4 synthesized by gingival inflammatory infiltrate lymphocytes presents an abnormal structure and function that will influence osteogenesis and aberrant bone regeneration in the alveolar bone despite the application of bone regeneration.

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Conflicts of Interest: The authors declare no conflict of interest.

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