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APPEARANCE AND DISTRIBUTION OF TISSUE REMODELLATION FACTORS IN THE HARD TISSUE OF PATIENTS AFFECTED BY CLEFT LIP PALATE

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Bone repair after surgical intervention on cleft lip palate (CLP) depends on the coordinated action of multiple tissue regeneration factors. We determined the relative number and appearance of tissue factors: matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-2 (TIMP-2), bone morphogenetic protein 2/4 (BMP 2/4), transforming growth factor beta (TGF-ß), Wnt3a protein (Wnt3a), Runt-related transcription factor 2 (Runx2), basic fibroblast growth factor (bFGF) and osteoprotegerin in hard tissue of CLP patients during first time surgical intervention. Fourty-three CLP patients with 24 bone and 36 cartilage samples were involved. Immunohistochemistry was used to assess the levels of tissue factors and the semi-quantitative census method was used for quantification of immunological structures. The increased amount of MMP-2 and bFGF positive cells was detected in the CLP group in cartilage and bone ($p < 0.05$), compared to the controls. A statistically significant ($p = 0.012$) increased amount of BMP 2/4 positive cells was found in cartilage of CLP patients, in comparison to the control group. Increased appearance of MMP-2, bFGF in hard tissue of the CLP patients indicates the predominance of tissue degradation. Increased number of BMP2/4 positive chondrocytes suggests improved cartilage growth and better regeneration in CLP patients.

Key words: *growth factors, human.*

INTRODUCTION

Cleft lip palate (CLP) belongs to serious facial deformations and occurs during embryogenesis, when the mesenchyme in the lateral palatine process fails to fuse with the intermaxillary segment (Norton, 2016, pp. 1–24). CLP incidence in the world is approximately one in 1000 newborns (Murray, 2002), in comparison to data from Latvia where incidence is one in 700 newborns (Akota *et al.,* 2001). The facial skeleton is composed of two types of tissue: cartilage and bone that are formed by chondrocytes and osteoblasts. Given that they share a common progenitor, it is important to understand which signalling pathways control the differentiation into bone and cartilage (Hartmann, 2006). Overall, CLP affects development of speech and hearing, breathing through the nose, olfactory function, eating, and is an aesthetic defect. Until this moment, surgical therapy is the method of choice for cleft palate correction, and the need for multiple surgeries is common (Tiwana *et al.,* 2018). There are many signal molecules, growth and remodellation factors, and genes involved in the development of facial clefts and the process of tissue healing after surgery. While studies about signal molecules and tissue factors are found in the literature, most have investigated animal tissue, and significantly less studies can be found about human tissue.

Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) are responsible for tissue remodellation in the process of craniofacial development. Additionally, degradation of extracellular matrix (ECM) by the MMPs stimulate cell proliferation and migration (Letra *et al.,* 2012). MMP-2 and MMP-9 are two proteases that have major activity in ECM degradation and are involved in degradation of type IV collagen as well as laminin — major components of basal membranes. In addition, they are also associated in a variety of processes like

bone remodelling, angiogenesis, immune response and tissue repair. Since these MMPs are secreted as proenzymes that require activation, their tissue distribution may not necessarily reflect the sites of enzymatic activity (Gkantidis *et al.,* 2012). Loss of MMP in mice impairs tooth root formation, alveolar bone remodelling, and periodontal ligament formation and integration into alveolar bone (Xu *et al.,* 2016).

TIMPs are also secreted into the ECM and have the ability to inhibit the activity of MMPs. In addition, TIMPs are considered to exert pluripotential effects on cellular behaviour such as cell growth, survival, migration, and differentiation, independently of their MMP neutralising functions. From the early stages of molar tooth development, location of TIMP-2 has been shown to be associated with the ECM presented in the jaw mesenchyme on the lingual side of the molar germ and along the lingual side of the dental basement membrane (Yoshiba *et al.,* 2007). Changed relations in the amount in favour of the TIMPs level can also be found in the tissue of the CLP (Verstappen *et al.,* 2006).

Bone morphogenetic proteins (BMPs) are multifunctional growth factors that mediate a variety of biological functions essential for gastrulation, organogenesis and embryonic and postnatal growth. BMPs also have an important role in the development of cranial neural crest, facial primordia, teeth, lips and palate. It is responsible for the migration of cells in the neural crest, which give rise to most of the craniofacial structures (Xuguang *et al.,* 2006). The BMPs signal controls cell division in the anterior palatal mesenchyme. Experiments on mice showed that BMP receptor inactivation in mesenchyme of maxillary bone could result in cleft lip and palate (Li *et al.,* 2013). Release of BMP-2 incorporated in an implant increased callus mineralisation and bone healing in the tibial bone (Faßbender *et al.,* 2014).

Transforming growth factor ß (TGF-ß) stimulates the production of collagen, fibronectin, proteoglycans and inhibits collagen degradation by both decreasing MMP activity and increasing the activity of TIMPs (Kumar *et al,* 2014, Chapt. 1, pp. 1–29). TGF-ß activates MMPs that are located in the midline epithelium of palatal bones and participates in the fusing of palatal bones (Li *et al.,* 2017). Bone ECM is the major storage site in the body for TGF-ß and it can be released and activated by resorbing osteoclasts, resulting in the inhibition of osteoclast formation, activity and stimulation of mature osteoblasts to produce bone matrix proteins, although the molecular mechanism by which the TGF-ß is released from ECM-bound stores remain unclear (Dallas *et al.,* 2002).

Fibroblast growth factors (FGFs) are members of a large family of signalling proteins. FGF signalling participates in all stages of development of palate, especially in the cell proliferation of the palate shelves. Sonic hedgehog (Shh) signalling is believed to be responsible for cell proliferation in the palate mesenchyme, and Shh signalling works together with the FGF signalling via coordinated epithelial-mesenchymal interactions (Weng *et al.,* 2018). FGFs also have proved to be important in the regulation of chondrocyte proliferation and initiation of chondrocyte hypertrophy (Logan *et al.,* 2004). Basic fibroblast growth factor (bFGF) is a member of the FGF family and bFGF signalling does not directly induce osteoblast differentiation, but is known to modulate osteoblast differentiation. However, the exact mechanism of the bFGF signalling in bone healing or regeneration has not been explained (Charoenlarp *et al.,* 2017).

Wnt3a is a signalling molecule that belongs to the Wnt protein family and is included in various skeletogenous processes, including cellular differentiation of the chondro-osseous progenitor cells, regulation of proper chondrocyte columns in growth plate and migration processes (Logan *et al.,* 2004). Signalling of the Wnt results with *Runx2* promoter activation and induces endogenous *Runx2* gene expression in pluripotent mesenchymal cells that later take a part into bone formation (Gaur *et al.,* 2005).

Runt-related transcription factor 2 (Runx2) belongs to the Runx family and is a transcription factor needed for the correct structure of osteoblast cells. The BMPs and the FGFs regulate transcription of Runx2. Runx2 also induces mesenchymal cell differentiation into immature osteoblasts until the final stage of the process during which maturity of the osteoblasts are inhibited (Komori, 2010; Dos Santos Pereira *et al.,* 2017). A study on Chinese patients showed that normal distribution of the Runx2 also might be a reason for cranial dysplasia due to the mutation in the Runt domain, which leads the impaired transaction activities of Runx2 (Zhang *et al.,* 2017).

Osteoprotegerin (OPG) is a protein of the Tumour Necrosis Factor (TNF) cell signalling protein family. It is the main osteoclastogenesis modulator, also known as inhibitor of terminal differentiation and activation of osteoclasts (Hsu *et al.,* 2005). OPG interacts with receptor activator of nuclear factor κB ligand (RANKL) by inhibiting binding of the RANKL to the RANK, and therefore resulting in osteoclastogenesis and inhibited bone resorption (Kwan *et al.,* 2008). Another study confirmed that the OPG during the osteoclastogenesis enhanced the size of osteoclasts, but attenuated their bone resorbing activity, suggesting that the OPG may play an auto regulatory role in a late phase of the osteoclastogenesis through the induction of apoptosis (Kang *et al.,* 2014). Promising results exist in the therapy for periodontitis affected tissue in rabbits. The study demonstrated that the periodontal ligament stem cells (PDLSCs) of gene-modified rabbits expressing human OPG achieved an earlier mineralisation and more bone formation, which might help to ensure the reconstruction of alveolar bone defect (Su *et al.,* 2015).

Based on all the information mentioned above, it is thought that remodellation factors have importance in morphopathogenesis of CLP. Also, based on the absence of these tissue factor's correlative research in hard tissue of CLP patients, the aim of our present study is to determine a relative number and appearance of the tissue factors (MMP-2, TIMP-2,

Table 1. Information on CLP patients

Patient	Age	Surgery procedure	Patient	Age	Surgery procedure
No. 1	5 years 7 months	Rhinoplasty	No. 22	13 years 9 months	Rhinoplasty
No. 2	6 years 7 months	Rhinoplasty	No. 23	13 years 9 months	Rhinoplasty
No. 3	7 years 3 months	Alveolar osteoplasty	No. 24	14 years 4 months	Rhinoplasty
No. 4	7 years 5 months	Rhinoplasty	No. 25	14 years 5 months	Rhinoplasty
No. 5	7 years 7 months	Rhinoplasty	No. 26	15 years	Rhinoplasty
No. 6	8 years 3 months	Rhinoplasty	No. 27	15 years 10 months	Rhinoplasty
No. 7	8 years 5 months	Alveolar osteoplasty	No. 28	16 years	Rhinoplasty
No. 8	8 years 7 months	Rhinoplasty	No. 29	16 years 3 months	Rhinoplasty
No. 9	8 years 11 months	Alveolar osteoplasty	No. 30	16 years 6 months	Rhinoplasty
No. 10	9 years 5 months	Alveolar osteoplasty	No. 31	16 years 7 months	Rhinoplasty
No. 11	9 years 6 months	Rhinoplasty	No. 32	16 years 10 months	Rhinoplasty
No. 12	9 years 11 months	Alveolar osteoplasty	No. 33	17 years	Rhinoplasty
No. 13	12 years 7 months	Rhinoplasty	No. 34	18 years	Rhinoplasty
No. 14	12 years 8 months	Rhinoplasty	No. 35	18 years	Rhinoplasty
No. 15	12 years 8 months	Rhinoplasty	No. 36	18 years 7 months	Rhinoplasty
No. 16	12 years 11 months	Rhinoplasty	No. 37	18 years 8 months	Alveolar osteoplasty
No. 17	12 years 11 months	Rhinoplasty	No. 38	18 years 8 months	Alveolar osteoplasty
No. 18	12 years 11 months	Rhinoplasty	No. 39	18 years 8 months	Alveolar osteoplasty
No. 19	13 years 1 month	Rhinoplasty	No. 40	18 years 8 months	Alveolar osteoplasty
No. 20	13 years 5 months	Rhinoplasty	No. 41	18 years 8 months	Alveolar osteoplasty
No. 21	13 years 9 months	Rhinoplasty	No. 42	21 years 1 month	Rhinoplasty

BMP 2/4, TGF-ß, bFGF, Wnt3a, Runx2, and OPG) in hard tissue (bone and cartilage) of CLP patients during the first plastic alveolar osteoplasty and rhinoplasty.

MATERIALS AND METHODS

Patients. Tissue samples were obtained during first time CLP correction surgeries, such as alveolar osteoplasty and rhinoplasty, in the Cleft Lip and Palate Centre of the Institute of Stomatology of the Rīga Stradiņš University (RSU). The research involved 43 patients with CLP between the ages of 5 years 7 months to 21 years and 1 month with 24 bone tissue and 36 cartilage tissue samples (see Table 1). Control material of bone was obtained from seven healthy patients from the facial cleft during unrelated surgical operations, due to the ethical difficulties related to receiving permission from parents for the usage of tissue (see Table 2). Due to the ethical considerations there was no possibility to obtain hyaline cartilage from children with facial cleft unrelated surgeries, and therefore cartilage tissue from trachea was obtained from the exposition of RSU Institute of Anatomy and Anthropology (AAI) from subjects between the ages of 20 to 40 years. The study was approved by the local Ethical Committee of the Rīga Stradiņš University.

Methods. The material from patients obtained during the first CLP correction surgery was fixated in transport test tubes with Stefanini (Zamboni) solution and delivered to the RSU AAI Laboratory of Morphology for further processing. After tissue fixation, the material was rinsed for 24 hours in Tyrode solution. An alcohol solution was used for tissue dewatering, and tissue was placed in xylene for 30 minutes for degreasing. The tissue was placed into paraffin for one and two hours for the hardening process. Tissue blocks were cut in 3 μ sections with a semi-automatic rotary microtome (Leica RM2245, Leica Biosystems Richmond Inc., United States). The sections were fixed on slides and dried in a thermostat, later re-dewaxed in xylene, and dehydrated in various alcoholic solutions.

Table 2. Information about the control group for bone

Patient	Age	Surgery procedure
No. 1	6 years 9 months	Tooth extraction
No. 2	9 years 9 months	Tooth extraction
No. 3	10 years 2 months	Tooth extraction
No. 4	10 years 5months	Tooth extraction
No. 5	11 years 7 months	Tooth extraction
No. 6	12 years 8 months	Tooth extraction
No. 7	14 years 5 months	Tooth extraction

Firstly, tissue sections were dyed with haematoxylin and eosin for a general overview. Afterwards, for the expression of genes and proteins we used the immunohistochemistry method. Tissue selections were stained and processed for the following antibodies: MMP-2 (code: ORB101049, rabbit, 1:400, Biorbyt USA), TIMP-2 (code: SC-21735, mouse, 1:50, Santa Cruz USA), BMP 2/4 (code: AF355, goat, 1:100, R/D Germany), TGFß1 (code: ORB7087, rabbit, 1:200, Biorbyt USA), bFGF (code: AB16828, rabbit, 1:200, Abcam GB), Wnt3a (code: AB1992, rabbit, 1:800, Abcam

GB), Runx2 (code: AB192256, rabbit, 1:250, Abcam GB) and OPG (code A0611, rabbit, 1:100, The Orbit USA).

For quantification of immunological structures we used the semi-quantitative census method (Pilmane, 1997) as follows: (0) – no positive structure was detected in the visual field (0 / +) – occasional positive structures seen in the visual field, (+) – few positive structures seen in the visual field, (+ / ++) – few to moderate number of positive structures seen in the visual field, (++) – moderate number of positive structures seen in the visual field, (++ / +++) – moderate to numerous positive structures seen in the visual field, (+++) – numerous positive structures seen in the visual field, (+++ / ++++) – numerous to abundant positive structures seen in the visual field, (++++) – abundance of positive structures seen in the visual field.

Statistical analysis of the data. For the statistical analysis the SPSS 21.0 software version (IBM Corp., Armonk, NY, USA) was used. Spearman's rank correlation coefficient (rs) was calculated for determination of correlations between values. The results were interpreted: rs = 0.4–0.59 – moderate, positive correlation, and rs = 0.6–0.79 – strong, positive correlation. The Mann-Whitney U-test was performed for the comparison of study groups; p value < 0.05 was considered to be statistically significant.

RESULTS

OPG. OPG-positive cells were detected in all bone and cartilage tissue samples from the CLP and the control group. The number of the OPG-positive osteocytes in both the control group and the CLP group was very variable, from occasional to numerous, but no statistically significant difference in median numbers was found between the CLP and the control group (U = 63.0; p = 0.610). The number of the OPG-positive chondrocytes in the control group varied from moderate to abundance, while in the CLP group it varied from occasional to abundance (Fig. 1a) (see Table 3). There was no statistically significant difference in the median numbers of the OPG positive chondrocytes between the CLP and the control group (U = 105.0; p = 0.051).

TGF-ß. TGF-ß positive osteocytes were observed in all bone and cartilage samples. Few to numerous TGF-ß positive osteocytes were present in the control group, while in the CLP group the number of TGF-ß-positive cells varied from occasional to numerous. No statistically significant difference in median value between the CLP group and the control group was found (U = 37.0; p = 0.061). The number of TGF-ß-positive chondrocytes in the control group varied from few to moderate number to abundance, while in the CLP group it varied from occasional to abundance (Fig. 1b) (see Table 3). However, there was no significant difference in median numbers of TGF-ß-positive chondrocytes between the CLP and the control group (U = 166.0; p = 0.480).

Runx2. Mainly there was an absence of Runx2-positive osteocytes in the control group in comparison to the CLP group, in which the absence of the Runx2 expression was observed only in three bone tissue samples, but relative number of Runx2-positive cells ranged up to few. There was no significant difference in the median numbers of Runx2-positive cells between the groups (U = 41.0; p = 0.073). The number of Runx2-positive chondrocytes in the control group varied from few to numerous, while in the CLP group it varied from occasional to abundance (Fig. 1c) (see Table 3). No statistically significant difference in median numbers between the groups was found (U = 182.5; p = 0.903).

Wnt3a. Wnt3a-positive cells were not detected in the specimens and the appearance of Wnt3a-positive osteocytes ranged up to moderate number in both the CLP and the control group (Fig. 1d) (see Table 3). The median number of Wnt3a-positive osteocytes was few in the control group and occasional to few in the CLP group, but there was no statistically significant difference between the groups in general (U = 66.5; p = 0.378). In comparison to bone specimens, Wnt3a-positive chondrocytes were found in all cartilage tissue samples. Moderate number to abundant Wnt3a-positive chondrocytes were present in the control group, while in the CLP group the number of Wnt3a-positive structures varied from few to abundance, showing no statistically significant difference in median value between the study groups (U = 154.5; p = 0.377).

TIMP-2. TIMP-2-positive cells were detected in all specimens. The number of TIMP-2-positive osteocytes in the control group varied from occasional to numerous, while in the CLP group it varied from occasional to moderate. No statistically significant difference in median value was found between the groups (U = 53.0; p = 0.169). The number of TIMP-2-positive chondrocytes in the control group varied from few to moderate number, to numerous to abundant number, while in the CLP group it varied from few to moderate number, to abundant (Fig. 1e) (see Table 3). No statistically significant difference between the groups was found (U = 160.5; p = 0.555).

bFGF. bFGF-positive cells were observed in all groups. The number of bFGF-positive osteocytes in the control group varied from few to numerous, while in the CLP group it varied from occasional to moderate number. The number of bFGF positive osteocytes was significantly higher in the CLP group than in the control group (U = 21.5; p = 0.002). The number of bFGF-positive chondrocytes in the control group varied from few to moderate number to moderate number, while in the CLP group it varied from few to abundant (Fig. 1f) (see Table 3). The number of bFG-positive chondrocytes was significantly higher in the CLP group in comparison to the control group (U = 79.0; p = 0.005).

MMP-2. Generally, there was an absence of MMP-2-positive osteocytes in the control group, where moderate number of MMP-2-positive structures was observed only in one specimen, while number of MMP-2-positive structures

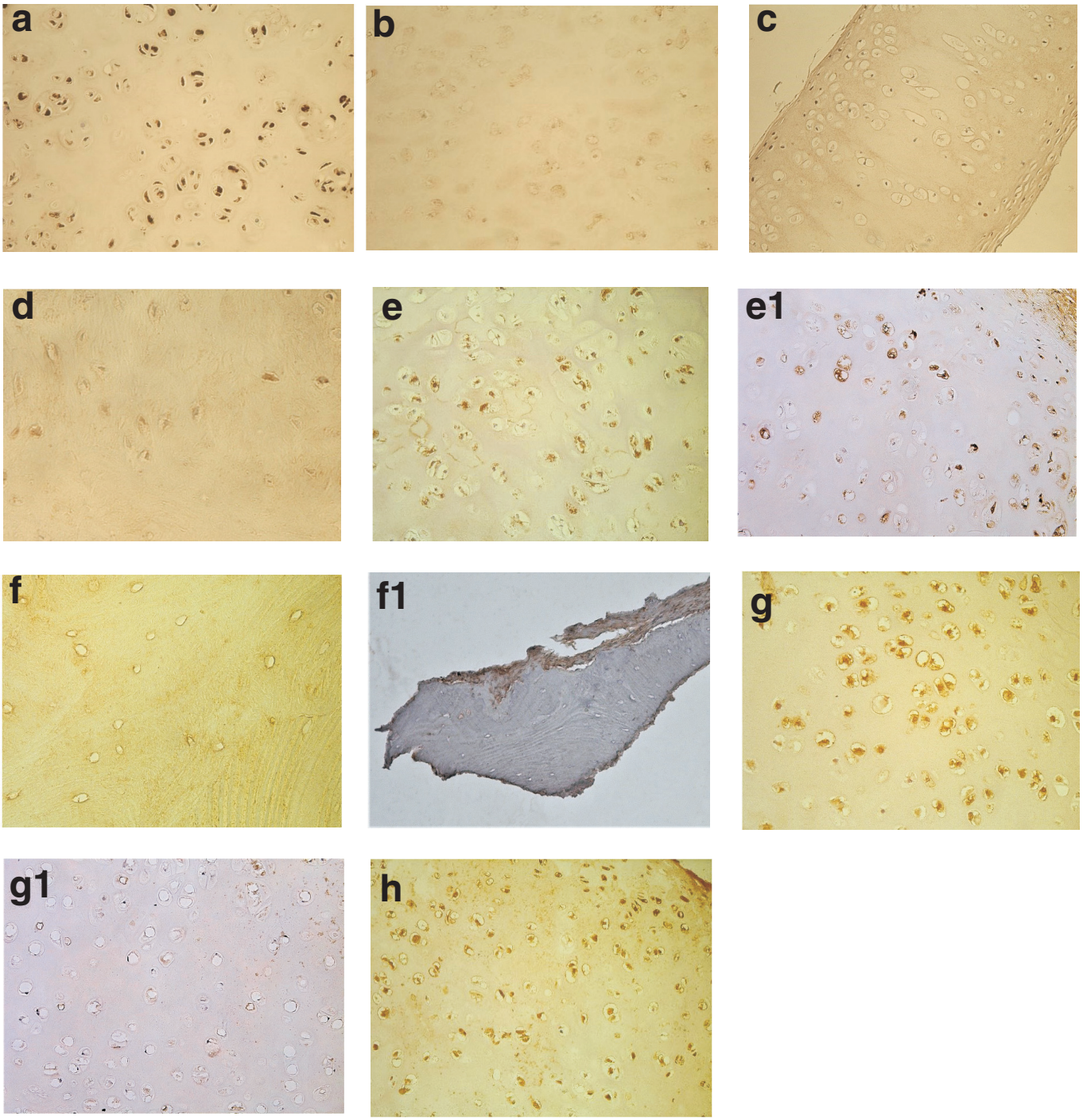

Fig. 1. Microphotographs of a relative number in different factors of hard tissue in cleft-affected patients. (a) Numerous OPG positive chondrocytes observed in the cartilage of 18 years old CLP patient, IMH, ×200. (b) Moderate number of TGF-ß-positive chondrocytes observed in the cartilage of a 13 year and 1 month old CLP patient, IMH, ×200. (c) Moderate number of Runx2-positive chondrocytes observed in the cartilage of a 13 year and 9 months old CLP patient, IMH, ×200. (d) Moderate number of Wnt3a-positive osteocytes observed in the bone of a 12 year and 8 months old CLP patient, IMH, ×200. (e) Numerous bFGF-positive chondrocytes observed in the cartilage of a 12 year and 7 months old CLP patient, IMH, ×250. (e1) Numerous to moderate number of bFGF-positive chondrocytes observed in the cartilage of the control patient, IMH, ×250. (f) No MMP-2-positive osteocytes observed in the bone of a 17 year old CLP patient, IMH, ×250. (f1) No MMP-2-positive osteocytes observed in the bone of a 10 year and 5 months old control patient, IMH, ×250. (g) Numerous BMP 2/4-positive chondrocytes observed in the cartilage of a 5 year and 7 months old CLP patient, IMH, ×250. (g1) Numerous BMP 2/4-positive chondrocytes observed in the cartilage of the control patient, IMH, ×250. (h) Numerous TIMP-2-positive chondrocytes observed in the cartilage of a 12 year and 11 months old CLP patient, IMH, ×200.

in the CLP group varied from occasional to numerous (Fig. 1g) (see Table 3). We observed a significantly higher number of MMP-2-positive osteocytes in the CLP group in comparison to the control group (U = 27.5; p = 0.011). The number of MMP-2-positive chondrocytes in the control group varied from few to moderate number to moderate number, while in the CLP group it varied from few to abundant. The number of MMP-2-positive chondrocytes was statistically higher in CLP group when compared to the control group (U = 91.5; p = 0.013).

Table 3. Relative number of factor-positive cells in cartilage and bone in the control group and in CLP patients

	OPG	TGF-ß	Runx2	Wnt3a	TIMP-2	bFGF	MMP-2	BMP2/4
Control cartilage	++/+++ - ++++	++/ - +++/++++	+ - +++	++ - ++++	+/++ - +++/++++	+/++ - +++	+/++ - +++	+/++ - +++
Median value	+++/++++	++ - ++/+++	++	++/+++ - +++	++/+++	++/+++	++	++
Patient cartilage	0/+ - ++++	0/+ - ++++	0/+ - +++/++++	+ - ++++	+/++ - ++++	+ - ++++	+/++ - ++++	0/+ - ++++
Median value	+++	++ - ++/+++	++	+++	++/+++	+++	++/++++	+++
Control bone	0/+ - +++	+ - +++	0 - ++	0 - ++	0/+ - +++	+ - +++	0 - ++	0-++
Median value	+	+/++	0	0/+ - +	+	+/++	0	0/+ - +
Patient bone	0/+ - +++	0/+ - +++	0 - +	+ - ++	0/+ - ++	0/+ - ++	0/+ - ++	0/+ - ++
Median value	+	++/+++ - +++	0/+	+	0/+	0/+	0/+	+

Abbreviations: OPG, osteoprotegerin; TGF-ß, transforming growth factor beta; Runx2, Runt-related transcription factor 2; Wnt3a, Wnt3a protein; TIMP-2, tissue inhibitor of metalloproteinase-2; bFGF, basic fibroblast growth factor; MMP-2, matrix metalloproteinase-2; BMP 2/4, bone morphogenetic protein 2/4. Median value – middle number in a sorted list of numbers. Quantification of structures: 0 – no positive structures in the visual field; 0 / + – occasional positive structures in the visual field; + – few positive structures in the visual field; + / ++ – few to moderate positive structures in the visual field; ++ – moderate positive structures in the visual field; ++ / +++ – moderate to numerous positive structures in the visual field; +++ – numerous positive structures in the visual field; +++ / ++++ – numerous to abundance positive structures in the visual field.

BMP 2/4. There was an absence of BMP2/4-positive osteocytes in two specimens of the control group, but the results ranged up to moderate number of positive cells, while in the CLP group it varied from occasional to moderate number, showing no significant difference in median value between the CLP and the control group (U = 60.0; $p = 0.232$). The number of BMP2/4-positive chondrocytes in the control group varied from few to moderate number to moderate number, while in the CLP group it varied from occasional to abundant (Fig. 1h) (see Table 3). A significantly lower number of BMP2/4 positive structures was observed in the control group in comparison to the CLP group (U = 91.0; $p = 0.012$).

Statistical data. Statistically significant ($p < 0.05$) strong ($r_s = 0.6–0.79$) correlations were found between MMP-2 and TGF-ß ($r_s = 0.602$; $p < 0.01$); Runx2 and Wnt3a ($r_s = 0.625$; $p < 0.01$) in CLP group bone (see Table 4).

Statistically significant ($p < 0.05$) moderate ($r_s = 0.40.59$) correlations were found between MMP-2 and BMP 2/4 ($r_s = 0.513$; $p < 0.05$); MMP-2 and bFGF ($r_s = 0.402$; $p < 0.05$); MMP-2 and Wnt3a ($r_s = 0.488$; p 0.05); MMP-2 and OPG ($r_s = 0.546$; p 0.01); TIMP-2 and Wnt3a ($r_s = 0.588$; $p < 0.01$); TIMP-2 and TGF-ß ($r_s = 0.464$; $p < 0.01$); TIMP-2 and BMP 2/4 ($r_s = 0.440$; $p < 0.05$); BMP 2/4 and Wnt3a ($r_s = 0.564$; $p < 0.01$); bFGF and TGF-ß ($r_s = 0.504$, $p < 0.05$); TGF-ß and Wnt3a ($r_s = 0.516$; $p < 0.05$); TGF-ß and OPG ($r_s = 0.578$; $p < 0.05$); OPG and Wnt3a ($r_s = 0.516$; $p < 0.05$) in the bone of the CLP group.

Statistically significant ($p < 0.05$) strong ($r_s = 0.6–0.79$) correlations were found between MMP-2 and bFGF ($r_s = 0.612$; $p < 0.01$); TIMP-2 and TGF-ß ($r_s = 0.663$; $p < 0.01$); TIMP-2 and BMP 2/4 ($r_s = 0.631$; $p < 0.01$); BMP 2/4 and TGF-ß ($r_s = 0.632$; $p < 0.01$); BMP 2/4 and OPG ($r_s = 0.683$; $p < 0.01$) in the cartilage of the CLP group (see Table 5).

Table 4. Summary of Spearman's rank correlation analysis. Strong and moderate relationships between numbers of positive factors in the bone of the CLP group are shown

Factor 1	Factor 2	r_s	p
MMP-2	TGF-ß	0.602	< 0.01
Runx2	Wnt3a	0.625	< 0.01
MMP-2	BMP 2/4	0.513	< 0.05
MMP-2	bFGF	0.402	< 0.05
MMP-2	Wnt3a	0.488	< 0.05
MMP-2	OPG	0.546	< 0.01
TIMP-2	Wnt3a	0.588	< 0.01
BMP 2/4	Wnt3a	0.564	< 0.01
bFGF	TGF-ß	0.504	< 0.05
TGF-ß	Wnt3a	0.516	< 0.05
TGF-ß	OPG	0.578	< 0.05
OPG	Wnt3a	0.516	< 0.05

For abbreviations see Table 3.

Table 5. Summary of Spearman's rank correlation analysis. Strong and moderate relationships between numbers of positive factors in the bone of the CLP group are shown

Factor 1	Factor 2	r_s	p
MMP-2	bFGF	0.612	< 0.01
TIMP-2	TGF-ß	0.663	< 0.01
TIMP-2	BMP 2/4	0.631	< 0.01
BMP 2/4	TGF-ß	0.632	< 0.01
BMP 2/4	OPG	0.683	< 0.01
MMP-2	TGF-ß	0.592	< 0.01
MMP-2	Wnt3a	0.484	< 0.01
TIMP-2	bFGF	0.457	< 0.01
TIMP-2	Runx2	0.464	< 0.01
TIMP-2	OPG	0.572	< 0.01
BMP 2/4	Wnt3a	0.496	< 0.01
BMP 2/4	Runx2	0.409	< 0.01
bFGF	TGF- ß	0.536	< 0.01
bFGF	OPG	0.578	< 0.01

For abbreviations see Table 3.

Statistically significant ($p < 0.05$) moderate ($r_s = 0.4–0.59$) correlations were found between MMP-2 and TGF-ß ($r_s = 0.592$; $p < 0.01$); MMP-2 and Wnt3a ($r_s = 0.484$; $p < 0.01$); TIMP-2 and bFGF ($r_s = 0.457$; $p < 0.01$); TIMP-2 and Runx2 ($r_s = 0.464$; $p < 0.01$); TIMP-2 and OPG ($r_s = 0.572$; $p < 0.01$); BMP 2/4 and Wnt3a ($r_s = 0.496$; $p < 0.01$); BMP 2/4 and Runx2 ($r_s = 0.409$; $p < 0.01$); bFGF and TGF-ß ($r_s = 0.536$; $p < 0.01$); bFGF and OPG ($r_s = 0.578$; $p < 0.01$) in the cartilage of the CLP group.

DISCUSSION

In this study, we sought to understand the mechanisms that participate in bone and cartilage remodelling in CLP patients and possible outcomes related to tissue healing after a surgical intervention. Bone repair after surgical intervention depends on the coordinated action of multiple tissue regeneration factors. The numbers and distribution of the tissue factors that may have a role in regeneration have not been widely investigated.

OPG is the main osteoclastogenesis modulator, also known as inhibitor of terminal differentiation and activation of osteoclasts (Hsu *et al.,* 2005). Periodontal ligament stem cells of rabbits expressing human OPG achieved earlier mineralisation and gained more bone formation that could help to reconstruct alveolar bone defect (Su *et al.,* 2015). There is evidence that endogenous OPG protects against cartilage destruction in osteoarthritis (Shimizu *et al.,* 2007). In contrast, Takegami *et al.* (2017) found that OPG may play a part in the opposite mechanism — progression of the cartilage degeneration that is seen in inflamed intravertebral discs (Takegami *et al.,* 2017). Although our study showed no statistically significant difference between the CLP group and the control group, more pronounced numbers of OPG positive cells were found in chondrocytes of CLP patient cartilage in comparison to bone. Correlations found between OPG and bFGF and BMP2/4 could indicate increased cartilage tissue proliferation instead of mineralisation.

Runx is necessary for the correct structure of osteoblast cells. It promotes bone mineralisation, osteoblast proliferation and induces mesenchymal cell differentiation into immature osteoblasts until the final stage of the process in which maturity of osteoblasts is inhibited (Dos Santos Pereira *et al.,* 2017). Our results indicated that there was no statistically significant difference between the CLP group and the control group, but the numbers of Runx2 positive structures in the bone and cartilage tissue was low, indicating reduced mineralisation potential of the hard tissue. Similar results were reported by Stricker *et al*. (2002) where Runx2 negative chicken embryos showed a decrease of endochondral ossiffication, which was caused by delay in chondrocyte maturation.

Wnt3a signalling increases bone formation through the stimulation of the development of osteoblasts, and inhibition of osteoblast and osteocyte apoptosis (Krishnan *et al.,* 2006). Variation in Wnt3a, which is one of factors responsible for neural crest cell development and migration, is associated with CLP (Chiquet *et al.,* 2008). In our study there was no statistically significant difference in Wnt3a positive cells between the CLP group and the control group, but we found a difference in relative numbers between bone and cartilage tissue. The lower number of Wnt3a-positive structures in bone could result in decreased mineralisation and ossification ability in comparison to cartilage where a moderate number of positive structures was observed. Therefore we assume that the initial ossification process could take place in the cartilage tissue for patients with CLP.

MMPs are responsible for tissue remodellation, degradation of ECM, cell proliferation, and tissue repair. Absence in MMP-2 can lead to loss of bone mineral density, cartilage destruction, and abnormal craniofacial development with decreases in osteoblast and osteoclast numbers *in vivo*, leading to impaired skeletal development (Mosig et al., 2007). A recent study demonstrated that loss of MMP in mice concludes with dentoalveolar tissue defect (Xu *et al.,* 2016). This concurs with another study that demonstrated increased MMP-2 expression in the bone healing process by osteoblasts and osteocytes (Itagaki *et al.,* 2008). In our study we observed a statistically significant ($p < 0.05$) increased amount of MMP-2 in CLP patients, both in cartilage and bone, compared to the control group, which could show that alveolar bone and nasal cartilage remodellation and healing is more pronounced in CLP patients. That could mean that tissue are more requisite for tissue repair and increased levels of MMP-2-positive cells could indicate the beginning of healing after surgical intervention. Strong correlation between MMP-2 and TGF-ß (rs = 0.602) in bone and MMP-2 and bFGF (rs = 0.612) in cartilage would support the process of increased tissue proliferation and remodellation.

TIMPs have the ability to inhibit the activity of MMPs and have effects on growth, survival, migration, and differentiation, and have an essential role in formation and maturation of the bone (Yoshiba *et al.,* 2007). TIMP-2 inhibits bone resorption and degradation of the organic matrix, which mainly consists of collagen I, therefore, remodelling bone. An imbalance of active MMPs over TIMPs is responsible for increased bone loss in pathological processes (Hill *et al.,* 1993). It has been found that osteoblasts and osteocytes in the incorporated bone also increasingly express TIMP2 (Hatori *et al.,* 2004). Polymorphism in TIMP2 is associated with the development of facial clefts (Letra *et al.,* 2012). In our research no statistically significant difference was found between the CLP group and the control group, but the increased levels of TIMP2 and strong correlations between TIMP2 and BMP2/4 (rs = 0.602) and TIMP2 and TGF-ß (rs = 0.602) in chondrocytes in comparison to osteocytes may suggest better healing potential in the cartilage.

TGF-ß acts as inhibitor for MMP activity and increases the activity of TIMPs, therefore it inhibits collagen degradation and stimulates collagen production *(*Mitchell, 2017). TGF-ß negatively regulates osteoclastogenesis by increasing levels of OPG in osteoblasts. The addition of TGF-ß as exogenous

applications in orthodontic treatment may be an approach to prevent periodontal damage (Yang *et al.,* 2014). In our study, there was no statistically significant difference between the CLP group and the control group in TGF-ß-positive cells. However, larger numbers of TGF-ß were found in cartilage, therefore indicating better tissue regeneration ability.

FGFs are potent angiogenic factors and have an important role in embryonic development and wound healing, angiogenesis, tissue repair and regeneration (Powers *et al.,* 2000). FGF signalling participates in all stages of palatogenesis, especially in cell proliferation during palatal fusion, and disturbance of this process in the central neural crest would cause a failure of palatal shelf fusion and would result in CLP (Weng *et al.,* 2018). A greater amount of new cementum deposits and new bone formation was observed six weeks after bFGF applications to bone defects created in dogs (Murakami *et al.,* 2003). In our study, we observed a statistically significant ($p < 0.05$) increase in amount of bFGF in the cartilage and bone of the CLP patients in comparison to the control group. These observations may suggest that increased proliferation and hypertrophy takes place in both chondrocytes and osteocytes in CLP patients, which could indicate a better possibility for successful wound healing.

BMP signal is critical for sceletogenesis due to regulation of chondrogenesis and osteogenesis. BMP signalling is involved in determination, migration, proliferation, differentiation and apoptosis of skeletal cells (Nie *et al.,* 2006). Disturbance in BMP-4 signalling pathways and genetic polymorphisms presented with diminished cell proliferation in maxillary process mesenchyme (Liu *et al*., 2005) and is clinically significant in the development and progression of CLP (Lin *et al*., 2008). It is concluded that BMP applications could influence endochondral ossification, thereby accelerating fracture healing (Wang *et al.,* 2017) and also it has properties to increase proteoglycans and collagen production in the bone at the place of osteoplasty (Sekiya *et al.,* 2005). BMP-2 has the ability to induce mesenchymal cells to differentiate into osteoblast cells. Herford *et al.* showed that premaxillary osseous clefts, repaired with the material filled with BMP-2, could result in efficacious bone repair without autogenous bone carrier graft (Herford *et al.,* 2007). There was no statistically significant difference found between the CLP group and the control group for bone, but a statistically significant ($p = 0.012$) increased amount of BMP 2/4 was found in the cartilage of the CLP patients in comparison to the controls. We obtained statistically significant strong correlations between BMP2/4 and TIMP2 (rs = 0.631) and BMP2/4 and TGF-ß (rs = 0.632) which may suggest increased tissue proliferation and healing capacities in cartilage that exceeds bone healing.

CONCLUSIONS

Increased appearance of MMP2, bFGF positive structures in hard tissue of the CLP patients indicates the predominance of tissue degradation of mesodermal origin tissue.

Increased number of BMP2/4 positive chondrocytes suggests the elevation of cartilage growth and thus better regeneration in the cleft lip palate.

Slight predominance of OPG, Wnt3a, TIMP2, TGF-ß-positive structures in cartilage (although without the statistically significant difference) proves the tendency for higher cartilage plasticity, including regeneration in cleft affected tissue.

The observed low number of OPG and Runx2 positive cells in bone could indicate a reduced mineralisation potential in bone in the case of CLP.

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AUDU REMODELĀCIJAS FAKTORU SASTOPAMĪBA UN ATRADNE LŪPU UN AUKSLĒJU ŠĶELTŅU PACIENTU BALSTAUDOS

Kaulu dzīšana pēc lūpu un aukslēju šķeltņu ķirurģiskas korekcijas ir atkarīga no vairāku faktoru savstarpējas mijiedarbības. Šajā pētījumā tika novērtēts audu faktoru (MMP-2, TIMP-2, BMP 2/4, TGF-ß, Wnt3a, Runx2, bFGF un OPG) relatīvais daudzums šķeltnes skartu pacientu un kontroles grupas balstaudos. Pētījumā tika iekļauti 43 lūpas un aukslēju šķeltnes skarti pacienti un analizēti 24 kaulaudu paraugi un 36 skrimšļaudu paraugi. Kontroles grupas materiāls tika iegūts ar šķeltni nesaistītu operāciju rezultātā. Imūnhistoķīmiski noteikto un analizēto struktūru kvantifikācijai tika izmantota puskvantitatīvā skaitīšanas metode. Šķeltnes pacientu kaulaudos un skrimšļaudos tika novērots palielināts MMP-2 un bFGF pozitīvo šūnu daudzums, salīdzinot ar kontroles grupu, kas norāda uz audu degradācijas predominanci šķeltnes pacientu balstaudos. BMP 2/4 pozitīvo šūnu daudzums šķeltnes pacientu skrimšļaudos bija statistiski ticami lielāks (p = 0,012) nekā kontroles grupas pacientiem. Palielināts BMP2/4 pozitīvo šūnu daudzums šķeltnes skartu pacientu skrimšļaudos norāda uz palielinātu skrimšļa augšanu un tādējādi labāku skrimšļa reģenerāciju lūpas un aukslēju šķeltnes pacientiem. Samazināts OPG un Runx2 pozitīvo šūnu daudzums šķeltnes pacientu kaulaudos varētu norādīt uz samazinātu kaula mineralizācijas potenciālu. Aukslēju šķeltņu gadījumā audu proliferācijas un remodelēšanas procesi dominē pār mineralizācijas procesu.