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FUNCTIONAL MORPHOLOGY
OF TISSUES IN ONTOGENETIC
ASPECT IN CHILDREN
WITH COMPLETE BILATERAL
CLEFT LIP AND PALATE

Summary of the Doctoral Thesis
for obtaining the degree of a Doctor of Medicine

Speciality – Morphology

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INTRODUCTION

Facial cleft is one of the most common congenital craniofacial dysplasia which causes aesthetic and functional disorders in children (Maclean *et al.*, 2009). Of all cases of clefts, the nonsyndromic ones make 70–90%, while the syndromic ones – 10–30% (Jugessur *et al.*, 2009; Ray and Niswander, 2012). Clefts which affect the lip, alveolar bone and palate, can be unilateral and bilateral. In Latvia, on average, one child is born with a cleft per 700–800 newborns, or, on average, 1.2 – 1.4 per 1000 live births (Akota *et al.*, 2001).

In the morphopathogenesis of the upper lip and secondary palate merging are involved several precisely coordinated processes: cell proliferation, differentiation, migration, programmed cell death, synthesis of extracellular matrix (ECM), formation of the basis of local tissue protection factors, which are regulated by different signal molecules and the growth factors. Certain genes and the external factors can cause the lack of a specific signal molecule and/or growth factor, or their excessive presence, resulting in the development of facial clefts (Meng *et al.*, 2009).

Facial cleft is one of the congenital pathologies, the treatment of which has always been very topical. It is complex, long-lasting and expensive. As a result of multi-stage operations, the tissues may heal leaving a permanent defect, which can negatively influence the growth of craniofacial tissues. Wound healing and tissue remodelling are affected by the growth factors, transcription factors and their respective receptors, gene proteins, angiogenesis-promoting factors, matrix metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs) and other signal molecules.

Nowadays, using morphological and immunohistochemical methods, we can study the morphopathogenesis processes at the cell level in the cases of embryonic pathologies, being, undoubtedly, an important aspect.

The aim of this research was to a morphological study of specific signal molecules and cell deaths in cleft-affected tissues, as well as identification of those factors, which most essentially are describing morphopathogenesis of complete bilateral cleft lip and palate in the ontogenetic aspect.

In order to do the study, the following **objectives** were set:

1. To investigate the relative amount and distribution of matrix metalloproteinases MMP-2, MMP-8, MMP-9 and their related endogenous tissue inhibitor TIMP-2, TIMP-4 in the cleft-affected tissues in children with complete cleft lip and palate, using the immunohistochemical method.

2. To determine the relative amount and distribution of gene proteins MSX1, IRF6, PAX9 and RYK in cleft-affected tissues in children with complete cleft lip and palate, using the immunohistochemical method.

3. To assess the relative amount and distribution of growth factors VEGF, TGF β 3, a transmembrane phosphoglycoprotein CD34 and innervation factor PGP 9.5 in cleft-affected tissues.

4. To assess the relative amount and distribution of cell proliferation marker Ki-67 and nestin in cleft-affected tissues in children with complete cleft lip and palate, using the immunohistochemical method.

5. To determine the relative amount and distribution of apoptotic cells in complete cleft-affected tissues, using TUNEL method.

6. To determine the relative amount and distribution of the alveolar bone remodelling-affecting factors OC, OP, OPG, MMP-2, TIMP-2, BMP2/4, TGF β 3 in cleft-affected tissues in children with complete cleft lip and palate, using the immunohistochemical method.

7. To study the distribution and relative amount of specific proteolytic enzymes, growth factors, transcription factors, gene proteins, angiogenesis-affecting factors, alveolar bone remodelling-affecting factors, cell proliferation marker, innervation factor and cell deaths in unaffected control subjects.

8. To perform statistical analysis and to determine the possible cross-correlation of the acquired morphological data.

Hypotheses of study

1. The developmental process of bilateral cleft lip and palate is characteristic of local expression variability of certain single factors.
2. Complete bilateral cleft-affected local response reaction in regeneration and degeneration processes is determined by the relative amount and expression of specific signal molecules.

Novelty of study

Despite quite a lot of world research on the animal models and single studies on human tissues, there are still not clear data acquired of local different proteolytic enzymes, growth factors, transcription factors, gene proteins, angiogenesis-affecting factors, innervations, cell proliferation markers and the distribution and location of apoptotic cells in children with the most severe type of cleft – complete bilateral cleft lip and palate in the ontogenetic aspect.

For the first time a morphological study is being carried out on the complex of complete bilateral cleft lip and palate-affected tissues of different specific signal molecules in the ontogenetic aspect. All in all, in the most severe type of the cleft – complete bilateral cleft lip and palate was found the presence of decreased transcription factor IRF6, MSX1 and PAX9, the growth factor TGF β 3 and VEGF, as well as the decreased cell proliferation and apoptosis in the soft tissues, but in the supporting tissues - the decreased OPN, OPG, MMP-2, TIMP-2, BMP2/4 and TGF β 3 presence. It was conclusively proved, that morphological tissue changes were more severe in patients with bilateral clefts. During repeated operations, one and the same patients we were identified an

increased MSX1, MMP-9, TIMP-4, TGFβ3, Ki-67 and decreased VEGF expression.

Individual contribution

The author of the current work has performed all stages of research: planning of research work, selecting the immunohistochemical markers, collecting the study material, visiting the cleft operations, acquiring scientific data and doing statistical analysis. The author has participated in performing immunohistochemical reactions and using the TUNEL method and has done immunohistochemical visualization, analyzing each tissue sample under the light microscope. She is the author of all microphotographs included in the scientific work.

Ethical aspects

The research work was done in accordance to Helsinki declaration and the permission of Rīga Stradiņš University Ethics Committee: Rīga Stradiņš University Ethics Committee decisions on May 22, 2003 and January 17, 2013.

Structure and volume of the doctoral thesis

The doctoral thesis is written in the Latvian language. It is of the classical structure and consists of 8 parts: introduction, literature review, material and methods, results, discussion, conclusions, references and appendices. The doctoral thesis comprises 209 pages, including 40 tables, 36 pictures and 98 microphotographs. The list of references consist of 321 sources.

1. MATERIAL AND METHODS

1.1 Morphologically studied material

Material for morphological studies was collected at RSU Institute of Stomatology, Lip, Palatal and Facial Cleft Centre within the period from 2003 till 2015. Lip, alveolar process and palatal cleft-affected tissues were collected during the primary lip, soft and hard palate plasty, as well as performing osteoplasty with bone autotransplantation of alveolar process.

To do routine histological and immunohistochemical methods, the study material was processed at RSU AAI Laboratory of Morphology.

In the study of complete cleft lip and palate 46 patients were included: 22 children had complete bilateral cleft lip and palate, 24 children – complete unilateral cleft lip and palate. The total number of morphological material units, obtained from patients with bilateral cleft lip in primary plasty was 16, during soft palate plasty – 10, hard palatal plasty – 9, during osteoplasty – 11. The number of morphological material units in patients with unilateral cleft were 24 tissue samples, obtained from the primary cheiloplasty, and 12 tissue samples, obtained from osteoplasty.

Control material was obtained from upper lip frenuloplasty and tooth extraction. Soft tissue material was obtained at „RSU Institute of Stomatology” Ltd. In 2013 from children with upper lip frenum hypertrophy. For hard tissues the material was used from RSU AAI archives. During the time hard tissue material was taken, the control group patients – 4 girls and 3 boys were at the age from six years and nine months till 14 years and five months

Patient groups from whom the material for the examination was taken:

1 group – infants and children with complete bilateral cleft lip and palate after cheiloplasty;

2 group – children with complete bilateral cleft lip and palate after soft and hard palatoplasty;

3 group – infants and children with complete bilateral cleft lip and palate after osteoplasty;

4 group – infants with complete unilateral cleft lip and palate after cheiloplasty;

5 group – children with complete unilateral cleft lip and palate after osteoplasty;

6 group – control group, from which soft and hard tissues were obtained in operations not connected with clefts.

1.2 Morphological methods

1.2.1 Fixation of studied tissue material

24 hour-long tissue fixation was performed at RSU Institute of Stomatology, Lip and Facial Cleft Centre after cheiloplasty, soft palate plasty, hard palate plasty and osteoplasty. During the time of the operations mentioned the obtained tissue material was immediately placed into the further mentioned fixator. We have to add, that fixation solutions may be different, but the study material was fixed in Stefanini (Zamboni) solution, which was for the first time used by Stefanini et al. in 1967 for the fixation of spermatozoids (Stefanini *et al.*, 1967). After fixation the study material was taken to RSU AAI Laboratory of Morphology for rinsing with Tyrode solution for 24 hours, for dehydration in increasing concentration alcohol solutions, for degreasing in xylol for 30 min., and then one hour long immersing in paraffin I and two hours long in paraffin

II, immersing in melted paraffin, for solidification and further processing to do routine and immunohistochemical method.

1.2.2 Routine histological staining method

Three micrometers thin tissue cuts were prepared by means of semi-automatic rotation microtoma (Leica RM2245, Leica Biosystems Richmond Inc., USA) from the tissue material block obtained from each infant or a child during the operation. The cuts were put on the slides (code-6130603; Histobond®+, Paul Marienfeld GmbH & Co. KG, Germany), placed into the thermostat for drying, then deparaffinized in xylol, dehydrated, using different concentration alcohols, stained with hematoxyline and eosine, dropping the histological glue and covered with very thin 0.13-0.16 mm cover glass (code-H875.2; Carl Roth GmbH + Co, Germany) (Lillie *et al.*, 1976). Hematoxyline and eosine staining was necessary to have the review of the histological picture. Stained preparations were analyzed by light microscope (Leica DM500RB, Leica Biosystems Richmond Inc., USA).

1.2.3 Immunohistochemical method and reagents

Immunohistochemical method (Hsu *et al.*, 1981) was applied for detection of specific proteolytic enzymes, transcription factors, gene proteins, growth factors, cell proliferation markers, bone morphogenesis proteins, non-collagenous bone proteins and osteoprotegerine.

The following primary antibodies: MMP-2, MMP-8, MMP-9, TIMP-2, TIMP-4, MSX1, IRF6, PAX9, RYK, TGFβ3, nestin, CD34, VEGF, PGP 9.5, OC, OPN, OPG, BMP2/4 and Ki-67 were used in immunohistochemistry.

For the immunohistochemical staining protocol the following agents were used to visualize antigen-antibody reaction:

1. EDTA (pH 9.0) buffer (code-T0103; Diapath, Martinengo BG, Italy);
2. TRIS buffer (code-15-M106; Bio-Optica, Milano, Italy);
3. BIODER R decalcification solution (code-05-M03009; Bio-Optica, Milano, Italy);
4. Antibody Diluent (code-ab64211; Abcam, Burlingame, CA, USA);
5. HiDef DetectionTM reaction amplifier (code-954D-31; Sigma-Aldrich, Rocklin, CA, USA);
6. HiDef DetectionTM HRP polymer marker (code-954D-32; Sigma-Aldrich, Rocklin, CA, USA);
7. Goat ImmunoCruzTM ABC staining system (code-sc-2023; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), it includes biotin-containing secondary and tertiary antibodies, containing avidin and biotin-horseradish peroxidase solution;
8. DAB Substrate Kit (code-957D-30; Cell Marque, Rocklin, CA, USA);
9. Hematoxylin (code-ab143166; Abcam, Burlingame, CA, USA);
10. Histological glue Pertex[®] (code-00811; HistoLab[®], Gothenburg, Sweden).

Bone tissue prior to the further-mentioned processing was being treated for twenty-four hours in BIODER R decalcification solution.

From each block 3 micrometer thin tissue cuts were got, which were put on slides covered with adhesive silan, deparaffinized in xylol and hydrated in alcohol solutions with decreasing concentration. Rinsing was done 2 times every five minutes with TRIS buffer solution, boiling in EDTA buffer for 5 min in microwave. Endogenous peroxidase activity with 3% peroxidase was blocked for 10 min.. All antibodies used in research were diluted with Antibody Diluent.

HiDef Detection™ HRP polymer system was used for the mice or rabbit origin antibodies. In the case the current system was used after the primary antibody incubation and triple rinsing with TRIS buffer solution, HiDef Detection™ reaction amplifier was applied for 10 min at room temperature. After this processing, the preparations were rinsed three times for five minutes by TRIS buffer solution. After rinsing, using this polymer system, HRP chromogene was used for 5 min.

ImmunoCruz™ ABC staining system was used for antigens of goat origin. In the case ABC staining system was used, micropreparations were incubated by 1.5 % blocking serum TRIS buffer solution up to 1 hour at room temperature. Then there followed the incubation of preparations with primary antibodies for 1 hour at room temperature. After rinsing three times for five minutes in TRIS buffer solution, the cuts were incubated for 30 min with biotin-containing secondary antibody (biotinylated goat Ig). Then there followed repeated sample rinsing for five minutes in TRIS buffer solution for three times and incubation with tertiary antibody for 30 min at room temperature. Then rinsing with TRIS buffer solution followed. Further on DAB Substrate Kit was used for 10 min.

After chromogene, using both goat ImmunoCruz™ ABC staining system, and HiDef Detection™ HRP polymer system, hematoxyline – cell nuclei dye was used. After staining with hematoxyline the micropreparations were rinsed in distilled water and dehydrated in increasing concentration alcohols, transparenching in xylol and covering with the glue Pertex®.

The primary antibody in parallel cuts of the mentioned preparation was substituted by antibody diluent Antibody Diluent. These cuts were used as the negative control. Positive controls (in tissues which always have positive reaction) were prepared for each preparation series as well.

1.2.4 TUNEL method

During the operation the programmed cell death or apoptosis was assessed in the obtained soft tissue samples, using high sensitivity and specificity TUNEL (code: 11 684 817 910, In Situ Cell Death Detection Kit, Roche Diagnostics, Germany) kit' standardized set.

The following important reagents are contained in the set: final deoxynucleotidiltransferase, acquired from a calf's thymus, in nucleotide mixture buffer, anti-fluorescein antibodies and from the sheep-acquired Fab fragment, conjugated with horseradish peroxidase.

Firstly, for TUNEL reaction, the tissue samples were paraffinized, using xylol and decreasing concentration ethanol solutions (absolute, 95%, 90%, 80%, 70% ethanol), then, for about 10 min. they were rinsed in distilled water, and then in TRIS solution. Secondly, endogenous peroxidase activity was blocked with 3% Hydrogen peroxide, and tissue samples were rinsed in TRIS solution three times for 5 min. Thirdly, tissue samples were placed into EDTA buffer solution for 10 min and put into the microwave for 5 min, after that cooled at room temperature. Fourthly, preparations were rinsed in TRIS buffer solution and left in 0.1% cow's serum albumin phosphate buffer solution. In the next stage of work the tissue samples were covered with TUNEL mix (TUNEL Enzyme solution) for 1 hour at 37°C. After rinsing in TRIS buffer solution, the cuts were incubated two times for five minutes with horseradish peroxidase-containing reagent Convert-POD solution for 30 min at 37°C. Repeated samples were rinsed with TRIS buffer for five minutes, then the cuts were covered with DAB (10 min) solution for detection of peroxidase and rinsed in distilled water.

In the last stage of the protocol, the cell nuclei contrasting was done by Meyer hematoxyline, the tissue samples were dehydrated in increasing

concentration alcohol solutions, transparenising in xylol and closed in histological Pertex[®] glue, covered with a thin cover glass.

1.3 Semiquantitative method

The presence of immunohistochemical markers in tissues was assessed according to semiquantitative counting method (Pilmane *et al.*, 1998). Relative amount of positive structures were analyzed in ten, randomly chosen visual fields for each prepared tissue cut in the following way:

- 0 – no positive structures in the visual field;
- 0/+ – sepatate 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;
- ++++ – abundance positive structures in the visual field.

1.4 Statistical methods

To characterize the research groups in general, the conventional descriptive statistical methods (Teibe, 2007) were used. For the assessment of the research data, nonparametric statistical methods were applied. For the comparison of several unconnected groups, Kruskal–Wallis range dispersion analysis was used, which is Mann–Whitney test extension. For the comparison of five groups in pairs, there was used Bonferroni correction for the detection of p value significance level. By the range scale values Spearman’s correlation coefficient $|r|$ was calculated. The acquired results were interpreted: $|r| \leq 0.25$ –

weak correlation, $0.25 < |r| < 0.75$ – moderate correlation, $|r| \geq 0.75$ – close correlation. Results were considered to be statistically significant, if $p < 0.05$. Statistical analysis was done by SPSS 22 version (IBM Corp., Armonk, NY, USA).

2. RESULTS

2.1 Morphological findings

2.1.1 Routine histological data in control patients

From the reviewed preparations of control group patients used in research, in oral tissue samples of seven patients, obtained during upper lip frenoplasty, the histological findings were found to correspond to the generally-accepted norm. In the tissue samples, obtained from all control group patients teeth extraction, the morphological picture of supportive tissues was found to correspond to the generally-accepted norm as well.

2.1.2 Routine histological data in patients with clefts

The most pronounced morphological changes were found in the epithelium. In some patients its thickness differed, *i.e.*, in some patients we found a thin, atrophic, but in others – a thick epithelium with expressed basal layer cell hyperplasia, proliferation and vacuolisation of epithelial polymorphous cell. Besides, in some children with the cleft, the epithelial tissues were stretching into *lamina propria* located lower like long processes.

In patients with the cleft we could often visualize *lamina propria* with dense, and sometimes, chaotically located collagenous bundles of fibres, among which one could see single connective tissue cells, mainly, fibroblasts. In the epithelium of oral mucosa and in the connective tissue we observed also inflammatory cell infiltration, though not in all patients' tissue samples.

Quite often in the patients' tissue material, acquired during osteoplasty, we could observe the degenerative alveolar bone process. It was characterized by insignificant amount of osteons and their irregular distribution, as well as little amount of osteocytes.

2.2 Immunohistochemical profile and apoptosis

Control group patients

MMP-2 containing cells in the epithelium were of few to moderate (+/++) in the visual field. Only one in seven patients was found to have moderate (++) **MMP-2** containing connective tissue cells.

In the epithelium were seen separate (0/+) to moderate (++) of **MMP-8** positive cells. In the connective tissue the findings were similar, *i.e.*, **MMP-8** positive cells ranged from separate (0/+) to few (+) in the visual field.

For the majority of patients **MMP-9** positive epithelial cells ranged from separate (0/+) to moderate (++) cells in the visual field. **MMP-9** positive connective tissue cells ranged from separate (0/+) to moderate (++).

TIMP-2 containing epithelial cells ranged from separate (0/+) to moderate (++) cells. In two patients we found separate (0/+) **TIMP-2** positive connective tissue cells.

TIMP-4 presence in the epithelium ranged from separate (0/+) to moderate (++) positive cells in the visual field, while in the connective tissue the relative amount of **TIMP-4** positive cells ranged from separate (0/+) to few (+) amount.

In the epithelium were found few (+) to numerous (+++) **MSX1** positive cells in the visual field. **MSX1** in the connective tissue varied from few (+) to moderate (++) positive cells in the visual field.

In the epithelium **IRF6** positive cells were seen ranging from few (+) to moderate (++), but in the connective tissue – from separate (0/+) to numerous (+++) in the visual field.

All in all, in the epithelium we found moderate (++) **PAX9** positive cells. In three control group patients separate (0/+) **PAX9** containing connective tissue cells were found.

In the epithelium and connective tissue was found **RYK** containing cells, ranging from separate (0/+) to moderate (++) in the visual field.

In the epithelium and connective tissue **TGFβ3** containing cells were quite numerous (+++).

VEGF showed few (+) to moderate (++) endotheliocytes.

Immunohistochemically determined relative amount of **CD34** positive cells ranged from moderate (++) to numerous (+++) in the visual field.

All in all, **PGP 9.5** containing structures were separate (0/+) to numerous (+++) in the visual field.

In the epithelium there were found separate (0/+) to few (+) **Ki-67** positive cells in the visual field.

In the oral mucosa numerous (+++) cells contained **nestin**.

Apoptosis presence was seen in few (+) to moderate (++) epithelial cells.

OC presence in the tissues of control group patients was found in osteocytes and osteoblasts in all tissue samples. The relative cell amount, determined immunohistochemically, varied from moderate and numerous (++/+++) to abundance (+++++) positive cells in the visual field.

OPN varied from separate (0/+) to moderate till numerous (++/+++) positive osteocytes in the visual field.

OPG positive osteocytes varied from separate (0/+) to abundance (+++++) in the visual field.

The number of **BMP2/4** containing osteoblasts and osteocytes varied from few (+) to numerous (+++) in the visual field.

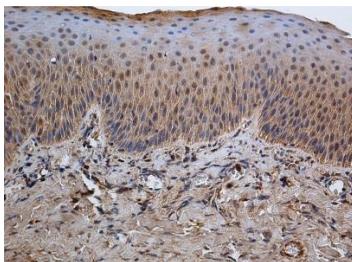
MMP-2, TIMP-2 varied from separate (0/+) to numerous (+++) positive osteocytes in the visual field.

TGFβ3 containing osteocytes varied from few (+) to numerous (+++) in the visual field.

Patients with clefts

MMP-2 finding in patients with complete bilateral cleft lip and palate

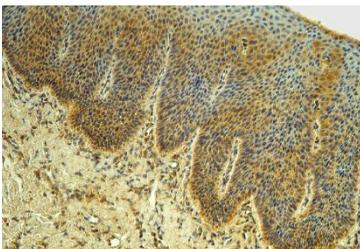
In the upper lip mucosal epithelium in all tissue samples were found moderate (++) to abundance (+++++) positive cells. MMP-2 positive connective tissue cells varied from few (0/+) to numerous (+++) in the visual field (2.1. microphotograph).



2.1. microphotograph. **Moderate (++) MMP-2 positive epithelial cells and connective tissue cells in oral subepithelium in 3 months old infant with complete bilateral cleft lip and palate after cheiloplasty. MMP-2 IMH, × 200**

In soft tissue epithelium we found separate (0/+) to moderate (++) MMP-2 containing cells in the visual field. Connective tissue MMP-2 positive cells varied from separate (0/+) to numerous (+++) in the visual field.

In hard palatal tissues MMP-2 containing cells varied from separate (0/+) to numerous (+++) in the visual field (2.2. microphotograph).

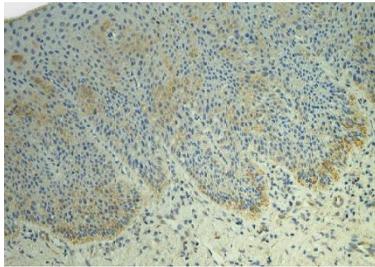


2.2. microphotograph. **Numerous (+++) MMP-2 positive cells in hard palatal mucosa in 3 years and 3 months old child with complete bilateral cleft lip and palate. MMP-2 IMH, × 200**

MMP-8 finding in patients with complete bilateral cleft lip and palate

In upper lip mucosal epithelium MMP-8 varied from few (0/+) to numerous (+++) positive cells in the visual field, while in the connective tissue from separate (0/+) to few (+).

We did not identify MMP-8 positive cells in soft palate in three patients. One patient had moderate (++) MMP-8 containing cells in the epithelium and numerous (+++) positive cells in the connective tissue (2.3. microphotograph). In hard tissue samples the relative amount of MMP-8 positive cells were from separate (0/+) to few (+) both in the oral mucosal epithelium, and in the connective tissue.



2.3. microphotograph. **Moderate (++) MMP-8 positive cells in soft palatal mucosa in 9 months old infant with complete bilateral cleft lip and palate. MMP-8 IMH, × 200**

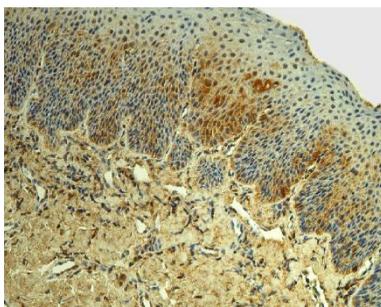
All in all, the relative amount of MMP-8 positive epitheliocytes and connective tissue cells in hard palatal mucosa varied from separate (0/+) to moderate (++) in the visual field.

MMP-9 finding in patients with complete bilateral cleft lip and palate

No MMP-9 positive cells were found in the upper lip mucosal epithelium in two patients tissue samples. In patients with complete bilateral

cleft lip and palate were fixed only separate (0/+) MMP-9 positive epitheliocytes and connective tissue cells.

In the material obtained from soft tissue plasty MMP-9 positive epitheliocytes varied from few (+) to numerous (+++) in the visual field. MMP-9 positive connective tissue cells finding was similar, though the mean amount of moderate immunoreactive structures in the connective tissue was higher (2.4. microphotograph).



2.4. microphotograph. **Moderate (++) MMP-9 positive epitheliocytes and numerous (+++) MMP-9 containing connective tissue cells in soft palatal mucosa in 9 months old infant with complete bilateral cleft lip and palate. MMP-9 IMH, × 200**

In the material obtained from hard palatal plasty, MMP-9 positive epitheliocytes in six patients varied from few (+) to numerous (+++), in three patients MMP-9 expression was not found. MMP-9 positive connective tissue cells in seven patients were found ranging from few (+) to numerous (+++), in two patients MMP-9 connective tissue cells were not visualized.

TIMP-2 finding in patients with complete bilateral cleft lip and palate

In upper lip mucosal epithelium TIMP-2 positive cells varied from few (+) to numerous (+++) in the visual field. TIMP-2 containing connective tissue cells varied from separate (0/+) to moderate (++) in the visual field.

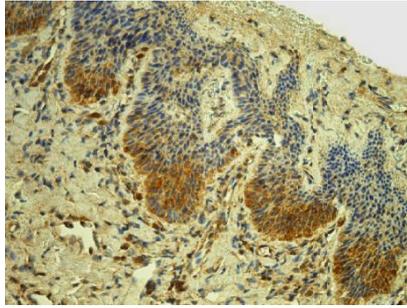
In three patients TIMP-2 containing cells in soft tissue samples were not visualized. In other patients tissue samples separate (0/+) to moderate (++) positive epithelial cells and connective tissue cells were found in the visual field.

In the tissue obtained during hard palatal plasty the results were the same as in the material of soft palate.

TIMP-4 findings in patients with complete bilateral cleft lip and palate

All in all, epitheliocytes of the upper lip mucosa contained few amount (+) of TIMP-4. In seven tissue samples *lamina propria* no TIMP-4 containing cell was identified. Eight cleft patients were identified TIMP-4 positive cells ranging from separate (0/+) to few (+) in the visual field.

In soft palatal tissue samples TIMP-4 positive epithelial cells in the visual field varied from few (+) to numerous (+++). At the same time in the underlying connective tissue fibroblasts, macrophages and single polymorphonuclear leucocytes contained moderate (++) TIMP-4 (2.5. microphotograph).

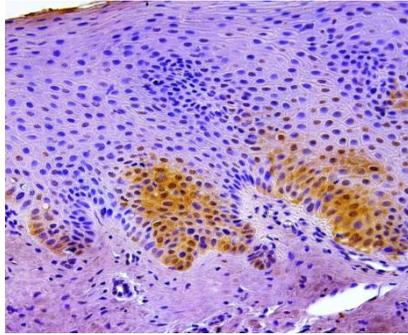


2.5. microphotograph. **Moderate (++) TIMP-4 positive epithelial cells and moderate (++) TIMP-4 containing connective tissue cells in 3 years and 3 months old infant with complete bilateral cleft after soft palatal plasty. TIMP-4 IMH, × 250**

In all the samples obtained from hard palatal plasty there were identified TIMP-4 positive cells both in the epithelium, and in the connective tissue, which varied in the visual field from moderate (++) to numerous (+++).

MSX1 findings in patients with complete bilateral cleft lip and palate

Oral mucosal epitheliocytes contained MSX1, and one patient were fixed separate connective tissue cells. MSX1 contained separate (0/+) to moderate (++) cells in submucosal epithelium (2.6. microphotograph).



2.6. microphotograph. **Few (+) MSX1 containing epithelial cells in the material obtained from cheiloplasty in 3 month old patient with complete bilateral cleft lip and palate. MSX1 IMH, × 200**

In the samples obtained from soft palatal plasty separate (0/+) to moderate (++) MSX1 positive epithelial cells were identified, while MSX1 in the connective tissue varied from separate (0/+) MSX1 to numerous (+++) positive cells in the visual field.

In hard palatal tissue material MSX1 containing epitheliocytes varied from separate (0/+) to numerous (+++) positive cells in the visual field, but MSX1 positive connective tissue cells – from separate (0/+) to moderate (++) in the visual field.

IRF6 findings in patients with complete bilateral cleft lip and palate

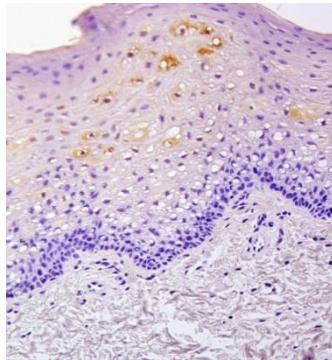
From 16 patients with complete bilateral cleft, IRF6 was identified in 11 patients in stratified squamous epithelium, ranging from separate (0/+) to few (+) positive cells in the visual field. IRF6 positive connective tissue cells were identified in all patients, except one, and their amount was ranging from separate (0/+) to moderate (++) in the visual field.

In soft palatal mucosal epithelium were seen separate (0/+) to numerous (+++), while in the connective tissue – few (+) to abundance (++++). IRF6 positive cells in the visual field.

The presence of IRF6 gene protein in the material obtained from hard palatal plasty was also seen in the epithelium and the connective tissue, in both tissue types there were found IRF6 containing cells from moderate (++) to numerous (+++).

PAX9 findings in patients with complete bilateral cleft lip and palate

In general, in the epithelium there was few (+) amount of epitheliocytes, containing PAX9 (2.7. microphotograph). It is interesting to say, that in neither of primary cheiloplasty obtained tissue samples there were identified PAX9 positive connective tissue cells.



2.7. microphotograph. **Few (+) PAX9 positive epithelial cells in 4 months old infant with bilateral cleft – cheiloplasty. PAX9 IMH, × 200**

Rare (0/+) to many (+++) PAX9 positive epithelial cells were observed in soft palatal mucosa. No PAX9 positive cells were visualized in the connective tissue.

Moderate (++) to abundance (++++) PAX9 positive cells were identified in hard palatal mucosal epithelium in the visual field. In one patient the hard palate *lamina propria* contained moderate (++) PAX9 positive connective tissue cells.

RYK findings in patients with complete bilateral cleft lip and palate

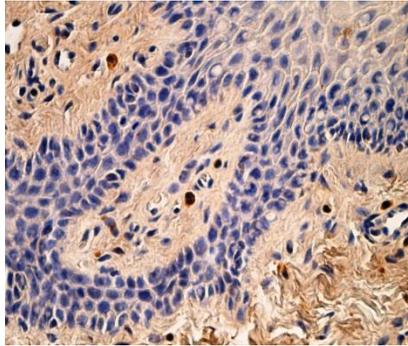
RYK presence in the upper lip mucosal epithelium was identified only in six patients with complete bilateral cleft; besides, in neither of patients there was found RYK expression in the lips *lamina propria*. It is interesting, that all six patients were identified separate (0/+) RYK containing epitheliocytes.

Separate (0/+) soft palatal epitheliocytes contained RYK protein. The relative amount of RYK expressing connective tissue cells in the material obtained from soft palatal plasty varied from separate (0/+) to moderate (++) cells.

RYK expression was found in eight patients hard palatal mucosal epithelium, separate (0/+) to abundance (++++) RYK positive epitheliocytes. In the material obtained from hard palatal plasty RYK expressing connective tissue cells relative amount varied from separate (0/+) to moderate (++) cells.

TGFβ3 findings in patients with complete bilateral cleft lip and palate

All in all, in patients with complete bilateral cleft the upper lip mucosal epithelium and the connective tissues separate (0/+) cells contained TGFβ3 (2.8. microphotograph).



2.8. microphotograph. **Separate (0/+) TGFβ3 positive connective tissue cells in 5 months old child with complete bilateral cleft lip and palate – cheiloplasty. TGFβ3 IMH, × 400**

In the material obtained during soft palatal plasty moderate (++) to abundance (+++++) TGFβ3 containing epitheliocytes and connective tissue cells were identified.

In the material obtained during hard palatal plasty the findings were similar, few to moderate (+/++) to abundance (+++++) TGFβ3 positive cells were visualized.

VEGF findings in patients with complete bilateral cleft lip and palate

Samples from twelve children, obtained during cheiloplasty contained few (+) to numerous (+++) positive endotheliocytes in the visual field.

In tissue samples, obtained during soft palatal plasty, separate (0/+) to few (+) VEGF containing endotheliocytes were found.

In all tissue samples, obtained during hard palatal plasty, VEGF positive endotheliocytes were identified; their number varied from separate (0/+) to moderate (++) in the visual field.

CD34 finding in patients with complete bilateral cleft lip and palate

All in all, the relative amount of CD34 positive cells was abundance (++++) in the visual field.

In the tissue samples, obtained from soft palatal plasty, CD34 positive cells varied from moderate (++) to abundance (++++) in the visual field.

In the tissue samples, obtained from hard palatal plasty, CD34 positive cells ranged from moderate (++) to abundance (++++) in the visual field.

PGP 9.5 findings in patients with complete bilateral cleft lip and palate

On average few to moderate (+/++) PGP 9.5 containing DNES structures were identified in the upper lip mucosa, from separate (0/+) to moderate (++) in the visual field.

In the samples, obtained from soft palatal plasty, neither PGP 9.5 positive structures were found in four patients. In four patients separate (0/+) nerve fibres in the connective tissue were identified, and in two patients tissue samples they were of few (+).

In the tissue samples, obtained from hard palatal plasty, there were identified separate (0/+) to numerous (+++) PGP 9.5 containing nerve fibres in the epithelium and subepithelium.

Ki-67 findings in patients with complete bilateral cleft lip and palate

Only one patient with complete bilateral cleft was identified the cell proliferation marker Ki-67 expression in the epitheliocyte nuclei. We have to mention, that in this patient tissue there were identified only separate (0/+) Ki-67 positive oral mucosal epitheliocytes.

In the tissue samples, obtained from soft palatal plasty, there were observed separate (0/+) to few (+) Ki-67 positive epitheliocyte nuclei.

In the material, obtained from hard palatal plasty, one patient was identified to have moderate (++), two patients - few (+), two patients – separate (0/+) Ki-67 containing cells in the visual field.

Nestin findings in patients with complete bilateral cleft lip and palate

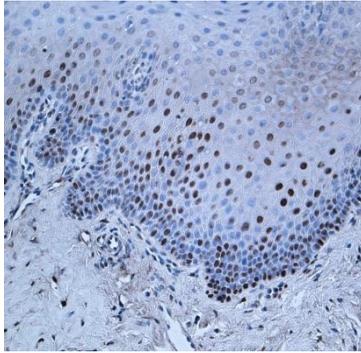
Altogether, moderate (++) nestin positive epitheliocytes were identified in the material, obtained from cheiloplasty.

In the soft palatal tissue samples, one could identify few (+) to numerous (+++) nestin positive cells in the visual field.

In the hard palatal tissue samples nestin containing cells were identified ranging from moderate (++) to numerous (+++) in the visual field.

Apoptosis findings in patients with complete bilateral cleft lip and palate

In patients with bilateral cleft in all tissue samples apoptotic cells varied from separate (0/+) to moderate (++) in the visual field (2.9. microphotograph).



2.9. microphotograph. **Moderate (++) apoptotic cells in a 4 months old child with bilateral cleft lip and palate – cheiloplasty. TUNEL, × 250**

OC finding in patients with complete bilateral cleft lip and palate

OC positive osteocytes in bilateral cleft lip and palate - affected tissues varied from few (+) to numerous (+++) in the visual field (2.10. microphotograph).



2.10. microphotograph. **Numerous (+++) OC containing osteocytes in 9 years and 2 months old child with bilateral cleft lip and palate – osteoplasty. OC IMH, × 250**

OPN finding in patients with complete bilateral cleft lip and palate

OPN positive osteocytes and osteoblasts varied ranging from separate (0/+) to few (+) in the visual field.

OPG findings in patients with complete bilateral cleft lip and palate

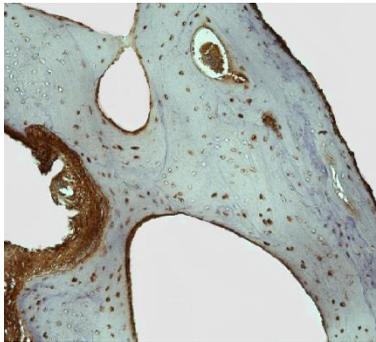
In patients with bilateral cleft, OPG positive cells varied from separate (0/+) to numerous (+++) in the visual field.

MMP-2 findings in patients with complete bilateral cleft lip and palate

In children with bilateral cleft, MMP-2 positive cells varied from separate (0/+) to numerous (+++) in the visual field.

TIMP-2 findings in patients with complete bilateral cleft lip and palate

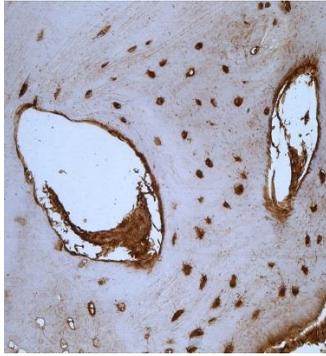
In patients with bilateral clefts, TIMP-2 positive osteocytes varied from separate (0/+) to numerous (+++) in the visual field. (2.11. microphotograph).



2.11. microphotograph. **Numerous (+++) TIMP-2 containing osteocytes in 7 years and 8 months old child with complete bilateral cleft lip and palate – osteoplasty. TIMP-2 IMH, × 100**

BMP2/4 findings in patients with complete bilateral cleft lip and palate

In patients with bilateral cleft there were identified separate (0/+) to numerous (+++) BMP2/4 positive cells in the visual field (2.12. microphotograph).



2.12. microphotograph. **Numerous (+++) BMP2/4 containing osteocytes in 8 years and 6 months old child with bilateral cleft lip and palate – osteoplasty. BMP2/4 IMH, × 200**

TGFβ3 findings in patients with complete bilateral cleft lip and palate

Altogether, moderate (++) TGFβ3 positive osteocytes were identified in children with complete bilateral cleft (2.13. microphotograph).



2.13. microphotograph. **Moderate (++) TGFβ3 containing osteocytes in 8 years and 6 months old child with bilateral cleft lip and palate – osteoplasty. TGFβ3 IMH, × 100**

2.3 Statistical data analysis

For cross-compliance closeness evaluation of factors identified in the control group tissue samples, we used Spearman's range correlation coefficient.

Statistically close, positive ($|r| \geq 0.75$) correlation was found:

$r_s = 0.938$; $p < 0.01$ – between ***TIMP-2* and *TIMP-4* positive epitheliocytes**;

$r_s = -0.933$; $p < 0.01$ – between ***MMP-9* positive connective tissue cells and *MMP-2* positive epitheliocytes**;

$r_s = 0.889$; $p < 0.01$ – between ***MSX1* positive connective tissue cells and *RYK* positive epitheliocytes**.

Statistical data of findings of cleft-affected tissues:

In total, the most severe cleft type – complete bilateral cleft lip and palate was found to have statistically significantly decreased transcription factor IRF6, MSX1 and PAX9, the growth factor TGF β 3 and VEGF presence, and also decreased cell proliferation and apoptosis in soft tissues, but in supportive tissues decreased OPN, OPG, MMP-2, TIMP-2, BMP2/4 and TGF β 3 presence ($p < 0.05$). We assertively identified, that morphological tissue changes were more severe in patients with bilateral cleft. During the repeated operations, one and the same patients were seen to have statistically increased MSX1, MMP-9, TIMP-4, TGF β 3, Ki-67 and statistically significantly decreased VEGF expression ($p < 0.05$).

Evaluation of immunohistochemically identified markers cross-compliance closeness data, using Spearman's range correlation coefficient

In the tissue material, obtained during cheiloplasty from infants with bilateral cleft lip and palate, were identified statistically significantly close,

positive correlation between MMP-8 and TIMP-4 in epithelium, and also between TIMP-4 in epithelium and TIMP-4 in the connective tissues. Most closer, positive correlations were identified in the tissue samples obtained from soft and hard palatal plasty tissue samples. In complete bilateral cleft lip and palate patients tissues, obtained during osteoplasty, there was identified close, positive correlation between BMP2/4 and MMP-2 in osteocytes.

In our study, we assessed also all immunohistochemically identified markers cross-compliance closeness in patients tissues in relation to the age in the tissues, obtained in repeated operations, using Spearman's range correlation coefficient.

Statistically significantly cose, positive ($|r| \geq 0.75$) correlation was found:

$r_s = - 0.882$; $p < 0.05$ – between the **age (per months)** and **TIMP-4** positive epitheliocytes;

$r_s = - 0.840$; $p < 0.05$ – between the **age (per months)** and **VEGF** positive structures;

$r_s = 0.868$; $p < 0.05$ – between the **age (per months)** and **MMP-8** positive structures;

$r_s = 0.939$; $p < 0.001$ – between the **age (per months)** and **PGP 9.5** positive structures.

3. DISCUSSION

All in all, the morphological findings of cell samples in the patients with complete unilateral and bilateral cleft lip and palate corresponded to the generally accepted norms. However, in a part of patients soft and hard tissue samples there were identified some nonspecific tissue changes under the light microscope, like epithelial atrophy, basal cell hyperplasia and proliferation, vacuolisation of *stratum spinosum* cell layer, as well as infiltration of inflammatory cells.

Studies on the possible facial cleft pathogenesis, postoperative wound healing and molecular mechanisms of tissue remodelling are mostly done on experimental animals in the laboratory. In the world there are widely studied different genetic variations of genes, using children's tissue samples of different populations. Not so many studies have been done on human embryos in relation to morphogenesis mechanisms of the facial and oral cavity development, as well as very few immunohistochemical studies of different tissue factors as to postnatally surgically obtained facial cleft-affected tissue morphology of infants and children in the ontogenetic aspect. Thus, the discussion can be mostly based on our data in correlation to the study results available in the literature, acquired from experimental animals. It is essential, that Bodo *et al.* has performed a significant research, identifying, that the cell phenotype of human cleft-affected cells after birth differed from the normal tissue cell phenotype (Bodo *et al.*, 1999). Due to this reason, studies with cleft-affected tissue samples, aquired postnatally, can be considered fit for the study of cleft etiopathogenesis, even if the cleft is tissue developmental disorder of embryonic aetiology

Lip and palatal morphogenesis include cell proliferation, differentiation, migration, programmed cell death, extracellular matrix synthesis and degradation, as well as the formation of the basis of local tissue protection

factors. The mentioned processes in embryonic tissues are coordinated by different signal molecules, growth factors, transcription factors and their receptors, tissue degradation enzymes and their endogenous inhibitors, as well as cell adhesion molecules. The absence of these factors in cells, or their excessive amount can cause the facial cleft in a child (Meng *et al.*, 2009).

MMP and TIMP immunohistochemical profile in the cleft-affected soft tissues. During the development of upper lip and palatal shelves, ECM remodelling, epithelial transformation in mesenchyme and facial tissue homeostasis postnatally is regulated by MMPs and tissue endogenous inhibitors TIMPs, which are identified by these enzyme expressions in the tissues of the mice. Just Mmp-2, Mmp-3, Mmp-7, Mmp-9, Mmp-13, Mmp-25 and Timp-1, Timp-2, Timp-3 expressions were assessed in the embryos of the mice during the merging of the palatal shelves. It is undeniable, that local changes in the relative amount of enzymes can produce tissue defects during the merging of lip and palatal shelves (Wiman *et al.*, 2000; Blavier *et al.*, 2001; Brown and Nazarali, 2010). MMPs and TIMPs are considered to be the possible nonsyndromic candidate genes for the cleft lip and palate. In many populations the genetic variations in MMPs and TIMPs genes are analyzed in relation to the facial clefts, but the results revealed for different populations and races are contradictory (Nikopensius *et al.*, 2011; Letra *et al.*, 2012; Letra *et al.*, 2014). Although the meaning of some types of MMPs and TIMPs in the development of the facial clefts is grounded, the local expression of these enzymes, however, has not been widely studied in the tissues, obtained postnatally from children with complete unilateral and bilateral cleft lip and palate in the ontogenetic aspect. In the current study the unilateral and bilateral cleft lip and palate-affected tissues there were assessed the relative amount and location of immunoreactive structures in MMP-2, MMP-8, MMP-9 un TIMP-2, TIMP-4, comparing them to the control group tissue samples.

In our study we found out a convincingly increased MMP-2 presence in the epithelium, which during the cheiloplasty was obtained from the children with complete bilateral cleft ($p < 0.001$), while MMP-2 in soft and hard palatal tissues was decreased ($p < 0.05$). We should mention, that MMP-2 presence in connective tissues in the control group patients was statistically significantly lesser than in the patients with clefts ($p < 0.05$). We, therefore, are of the opinion, that MMP-2 cell membrane associated MMP (MT1-MMP)/TIMP-2 signal-way is, undeniably, of importance in facial cleft-affected tissues in post-operative healing phases, i.e., in proliferation and remodelling, as well as in the scarring process and tissue homeostasis. It is quite possible, that by increased MMP-2 enzyme expression there is compensated the absence of some other enzyme functional activity. Altogether there was assessed the variability of the relative amount and location of MMP-2, therefore, it is possible that our patients may also possess MMP-2 genetic variations.

In 2002 the team of British scientists proved just a marked expression of MMP-2 in vivo during the palatal development. The discovery showed that by inactivating MMP-2, other – MMP-13 – MMP functions were affected as well (Brown *et al.*, 2002). In 2009 another team of scientists discovered, that the lack of heparinase was compensated by an increased MMP-2, MMP-9 and MMP-14 expression (Zcharia *et al.*, 2009). The study results undeniably confirm such a fact: one proteolytic enzyme function is impaired completely or incompletely, another tissue degradation enzyme is affected, and functional activity of endogenous inhibitor related to it, or the current enzyme function gets compensated by a different enzymes locally increasing expression in the tissues. Decreased MMP-2 expression in the wound healing process of the rats skin was found also in relation to an increased risk of the scarring (Dang *et al.*, 2003).

It is interesting, that MMP-2 functions are similar to MMP-9 functions, i.e., they degrade similar ECM components: gelatine, basal

membrane proteins and elastine (Ravanti and Kähäri, 2000). It has been proved, that MMP-9 essentially affects the wound healing, stimulating epithelial migration and regeneration (Mohan *et al.*, 2002). In our study, which was carried out with unilateral and bilateral cleft-affected tissues postnatally, we found a marked MMP-9 expression in milder cleft cases, but in more severe cases, i.e., in patients with complete bilateral cleft lip and palate, we found a significantly reduced MMP-9 presence. The same results have been acquired by Czech scientists with tissues from cheiloplasty in children from two to four months (Blaha *et al.*, 2013). In the experiments with animals it was found, that increased MMP-9 activity points to hypertrophic scarring during the wound healing, and an excessive collagen production in contrast to its decreased degradation (Hosokawa *et al.*, 2003; Manuel and Gawronska-Kozak, 2006). Considering the before-mentioned, we can admit, that the decreased MMP-9 presence in our patients upper lip tissues indicates to a greater scar formation risk in bilateral cleft cases.

We still have to add, that MMP-9 is increasingly expressed in the tissues of mice palate during the merging of palatal shelves (Wiman *et al.*, 2000). MMP-9 gene is localized 20q12.2 and is responsive for ECM basic component IV type collagen degradation, in such a way providing the cell migration (Zhang *et al.*, 1999). Considering MMP-9 functions, it is possible, that a reduced MMP-9 presence in the tissues causes incomplete basal membrane degradation, as a result, there is no epithelial transformation in mesenchyme, and consequently, no merging of lip and palatal shelves. Although there was seen a significant MMP-9 expression in the development of palatal development, there were not found any convincing studies on this enzyme involvement into nonsyndromic cleft aetiology. Letra *et al.* studied the importance of MMP-9 gene in the population of Brazilian inhabitants. MMP-9 1562 C/T polymorphism in the study was no convincingly identified in relation to nonsyndromic cleft lip and palate. The authors themselves refer it to

as having, an insufficient number of cases included in this study (Letra *et al.*, 2007). De Oliveira Demarchi and colleagues determined MMP-9 presence in palatal shelves in animals at different periods of embryonic development. On the 13th embryonic development day of the mice palatal development, MMP-9 expression was not visualized, but starting with the 14th till the 15th embryonic development day MMP-9 expression was identified in the epithelium of palatal medial margin. At this time, by degradation of basal membrane and extracellular matrix, there occurs the merging of palatal shelves in the mice. Thus, the idea was expressed as to the possible MMP-9 importance in the palatal ontogenesis (De Oliveira Demarchi *et al.*, 2010)

In the current study we found a decreased MMP-8 presence in the epithelial tissues of hard palate of complete bilateral clefts ($p < 0.05$), while in the tissues from cheiloplasty MMP-8 was found in a sufficient amount ($p < 0.05$), confirming that this enzyme is of importance in the remodelling of cleft-affected tissues. We found a close negative correlation with the child's age per months ($r_s = -0.868$; $p < 0.05$).

We could not find any convincing reports in the literature as to the importance of this enzyme in the palatal ontogenesis and in relation to lip and palatal clefts, as well as the variability of expression in relation to a child's age, and very few studies are published on the local expression of this factor in lip and palatal cleft-affected tissues in relation to wound healing, tissue remodelling and homeostasis processes, although MMP-8 is widely distributed in tissues and is expressed by different cells – epithelial cells, fibroblasts, macrophages, *etc.* (Van Lint and Libert, 2006). Tissues of craniofacial area are known to primarily develop from neural crest cells, and scientists have proved MMP-8 presence in different embryonic tissues, among them in the neural crest cells (Giambernardi *et al.*, 2001). Besides, Corpi *et al.* points, that MMP-8 is able to modulate the inflammatory process and collagen metabolism in the alveolar bone and oral mucosa, thus affecting the wound healing (Korpi *et al.*,

2008). In the study with human periodontal ligament cell samples, MMP-8 expression was proved to decrease with the increase of a man's age (Grzibovskis *et al.*, 2011).

In our study we assessed TIMP-2 presence in the material obtained from cheiloplasty of three to six months old infants. We found a statistically significant TIMP-2 decreased presence in the material obtained from soft and hard palatoplasty, in comparison to the acquired tissue samples from cheiloplasty of children with complete bilateral cleft lip and palate, besides, the acquired correlation was also preserved when calculating Bonferoni correction ($p < 0.05$), which indicated to the significance of the acquired result. TIMP-2 decreased expression was observed in the epithelial tissue. This fact can be explained by TIMP-2 strong inhibiting effect in the tissue degeneration in soft and hard palatal cleft-affected tissue, while an increased TIMP-2 presence might give evidence to upper lip wound healing of a greater risk of scarring. These data correspond to the assumption, that TIMP-2 – dependent, precisely coordinated MMP activity determines a successful palatal shelves merging, i.e., that a changed Timp-2 expression and functional activity hinder palatal shelves merging and the epithelial transformation in mesenchyme (Verstappen and Von den Hoff *et al.*, 2006).

In the study with human tissue samples there is shown TIMP-2 gene correlation with nonsyndromic cleft lip and palate in the European population, and the recent 2014 study identified TIMP-2 promoter polymorphism, revealing its relationship with the embryonally determined pathology (Nikopensius *et al.*, 2011; Letra *et al.*, 2014).

We visualized a significantly decreased TIMP-4 presence in children with complete bilateral cleft lip and palate, while TIMP-4 was rich with complete unilateral cleft lip and palate tissues ($p < 0.001$). However, TIMP-4 expression increased ($p < 0.001$) in the tissues obtained from palatoplasty. Convincingly significant TIMP-4 increasing expression was observed in the

connective tissues, acquired from repeated operations in one and the same patients with bilateral cleft lip and palate ($p < 0.05$). This finding can be explained by a, perhaps, decreased TIMP-2 expression in cleft-affected tissues is compensated by an increased local expression of a different inhibitor TIMP-4. It is interesting, that we found TIMP-4 close negative correlation with the child's age per months ($r_s = -0.882$; $p < 0.05$).

TIMP-4 inhibits different MMPs, and, consequently, this enzyme has, perhaps, a significant role in the morphogenesis of secondary palate. We have not succeeded in finding published data on TIMP-4 role in the facial and oral cavity development, its functional meaning in facial cleft-affected tissues in children, as well as on the variability of expression in relation to a child's age. It is interesting, that similarly to TIMP-4, also TIMP-4 functional activity is connected with MMP-2, *i.e.*, one of the functions of this protein functions is to inhibit MMP-2 and, under certain conditions, to activate proMMP-2 (Wang *et al.*, 2000). This protein inhibits also different MMPs, the role of which is proved also during the development of secondary palate, for instance, MMP-2, MMP-3, MMP-9 and MMP-13 (Koskivirta *et al.*, 2010). Besides, TIMP-4 indirectly regulates MMP-2 and MMP-4 expression in the growing gum and periodontitis-affected tissues. TIMP-4 is thought to have an essential importance in periodontitis pathogenesis (Kubota *et al.*, 2008; Nakasone *et al.*, 2009). In 2006, Zhang *et al.* proved a close correlation between TIMP expression and the organ changes due to the age (Zhang *et al.*, 2006).

Altogether, the relative amount and distribution of tissue degradation enzymes and their endogenous inhibitors, *i.e.*, in patients with complete unilateral cleft lip and palate, as well as in the control cases was greater rather than in patients with complete bilateral cleft lip and palate, which indicates to the importance of these proteins not only in the embryonic palatal development, as proved by many studies, but postnatally as well, when the cleft has been already developed.

The immunohistochemical profile of genes and their products in the cleft-affected tissues. Certain transcription factors, growth factors and genes regulate facial and oral cavity development, as well as affect MMP and TIMP, and other factor expression. The importance of the transcription factor IRF6 in the palatal ontogenesis has been discovered, and one can find convincing studies as to the essential role of the transcription factor IRF6 in the development of syndromic and nonsyndromic cleft lip and palate (Beaty *et al.*, 2010; Dougherty *et al.*, 2013). In a recent study there was identified the correlation between IRF6 gene V274I polymorphism and complete unilateral left side cleft lip and palate (Letra *et al.*, 2012). The data of the results of other authors relate IRF6 mainly to the cleft lip, cleft lip and/or palate and isolated cleft palate (Mostowska *et al.*, 2010; Nikopensus *et al.*, 2010; Larrabee *et al.*, 2011). In one of the studies the results are published on IRF6, as being the severity marker in patients with cleft lip and palate (Kerameddi *et al.*, 2015).

We identified a convincingly decreased IRF6 presence in children with the most severe type of the cleft – complete bilateral cleft lip and palate ($p < 0.001$), indicating to the relationship of this factor to the severity of the cleft. It has to be mentioned, that there was found a close, positive correlation between IRF6 positive epithelial cells and connective tissue cells in the tissues acquired from soft and hard palatoplasty of children with bilateral cleft ($r_s = 0.802$; $p < 0.05$; $r_s = 0.818$; $p < 0.05$). During repeated operations the differences in one and the same patients in the presence of the transcription factor were not found. This finding indicates to the even more closer relationship of this factor to the severity of the cleft despite the anatomic localization and the kind of the operation of the cleft-affected tissues. We found an increased IRF6 presence in the oral cavity epithelial cells, connective tissue cells and the cells of small salivary glands in children with complete unilateral cleft lip and palate ($p < 0.001$). It is interesting, that in a recent study there were

identified important IEF6 functions – regulation of epitheliocyte proliferation and formation of oral periderma during the embryonic development (Ke *et al.*, 2015). It was proved, that IRF6 was intensively expressed in facial shelves prior to primary palatal morphogenesis (Washbourne and Cox, 2006). Provisionally, in the mice there were found IRF6 expression in the palatal medial margin epithelium during the merging of secondary palatal shelves, in dental germs, hair follicles and the skin (Kondo *et al.*, 2002).

We also analyzed the local presence of RYK gene protein in the cleft-affected tissues. There are not so many studies on the probable importance of RYK gene in cleft lip and palate etiopathogenesis and in the proliferation of cleft-characteristic phenotypic cells, differentiation and polarity regulation in the postnatal period. Comparing it to the control group patients ($p < 0.001$), we saw a decreased amount of RYK immunoreactive structures in the connective tissues acquired from the cleft-affected tissues during cheiloplasty in patients with bilateral cleft, which indicates to a probable importance of this factor in the etiopathogenesis of facial clefts. In patients with complete bilateral cleft lip and palate after cheiloplasty, less positive structures were visualized, while in the tissue samples acquired in soft and hard palatoplasty an increasing positivity ($p < 0.05$) was observed. This observation can be explained by a decreased cell differentiation potential in the lip cells, in comparison to the tissues acquired in soft and hard palatoplasty.

RYK gene is an essential factor in affecting the morphogenesis of facial and oral cavity structures. Watanabe *et al.* indicates to the possible missense mutation 1355G>A (Y452C) in RYK gene and the relationship of rare single nucleoid polymorphism with the cleft lip and/or palate development in Vietnamese children population, and the cleft lip and/or palate, or isolated cleft palate development in Japanese children population (Watanabe *et al.*, 2006). Studies on RYK gene protein localization and in situ hybridization prove the expression of this factor not only in embryonic craniofacial tissues, but

postnatally as well. It was also proved, that an insufficient functional activity of RYK gene in the mice embryos causes craniofacial defects, it is, complete clefts and also skeletal defects (Halford *et al.*, 2000).

One of the most important factors influencing the facial, skeletal and dental development is the transcription factor MSX1, which is widely expressed in the craniofacial tissues and is promoting the development of anterior palatal part (Bush and Jiang, 2012). Both MSX family representatives – MSX1 and MSX2 are responsible for the facial and oral cavity development (Dai *et al.*, 2014). In the studies with animals it was found out, that this gene regulates ETM and induced apoptosis (Hwang *et al.*, 1998; Lallemand *et al.*, 2009). Nassif *et al.* also proved the importance of MSX1 in the mice facial tissue remodelling (Nassif *et al.*, 2014). Also in the recently done studies there was shown a possible relationship with MSX1 gene's genetic variations and nonsyndromic cleft lip and/or palate (Cardoso *et al.*, 2013; Ma *et al.*, 2014; Gurramkonda *et al.*, 2015).

The acquired study results revealed a decreased MSX1 positive epithelial cell presence in patients with complete bilateral cleft lip and palate after lip, soft and hard palate plasty, in comparison to the patients with a unilateral cleft lip and palate. The same fact was observed also in the control group patients ($p < 0.05$). It is interesting, that also the relative amount of MSX1 positive connective tissue cells in the material taken from patients with bilateral cleft after cheiloplasty was lesser ($p < 0.001$), while in the tissue samples from soft and hard palatoplasty, the relative amount of cells convincingly increased ($p < 0.001$). It shows, that MSX1 is essential morphology characterizing transcription factor of bilateral cleft lip and palate-affected tissues. Undeniably, this factor is involved in the most severe cleft-type tissue remodelling. While analyzing the relative amount and distribution of four gene proteins in one and the same patient in the ontogenetic aspect, there was found only MSX1 increasing presence in the repeatedly taken tissues

($p < 0.05$), which proves the more convincing reliability of this proteins involvement into the regulation of the most severe cleft type cell proliferation, differentiation and migration.

Genetic study results give evidence to the possible PAX9 gene relationship to nonsyndromic cleft lip and/or palate (Lee *et al.*, 2012; K uchler *et al.*, 2014).

In the bilateral cleft-affected tissues, obtained after cheiloplasty, we saw significantly lesser amount of PAX9 containing epitheliocytes and connective tissue cells, rather than in unilateral cleft-affected cells ($p < 0.001$). There was observed an increasing PAX9 presence in the epithelium of soft and hard palate in bilateral clefts ($p < 0.001$). It is interesting, that in this study there was found the association between PAX9 and MSX1 containing epithelial cells and connective tissue cells in children with complete unilateral cleft lip and palate ($p < 0.05$). Besides, PAX9 containing epithelial cells correlated with PAX9 containing connective tissue cells in children with the material obtained from hard palatoplasty of bilateral cleft ($p < 0.05$). It could be interpreted as a probable approval of Nakatomi *et al.* theory, ascerting, that by mutual interacting PAX9 and MSX1 genes regulate the facial tissue morphogenesis, and the reduced presence of these gene protein in a man can increase the risk of cleft development (Nakatomi *et al.*, 2010).

Assessing the mutual associations between PAX9 and IRF6 gene protein presence in the tissues, there were found significant both close, and moderately close positive correlations in the cleft-affected tissues. This fact points to Song *et al.* assumption, that the gene interactivity of PAX9 and IRF6 is potentially important in the cleft etiology (Song *et al.*, 2013). In conclusion we have to emphasize, that in this study all four gene protein (PAX9, RYK, MSX1, IRF6) mutual correlation in the cleft-affected tissues was assessed holistically. We found a close, positive correlation in the hard palatal cleft-affected tissues between IRF6 and RYK containing structures, which gives evidence to the

probable interrelationship of this gene in the palatal morphogenesis. Since we could not find any published data as to this assumption, we estimate our finding as an innovative discovery.

Apoptosis and cell proliferation in soft tissues of the cleft. The cleft-affected tissues are characterized both by cell proliferation, and apoptosis. The relative amount of the apoptotic cells in patients with unilateral cleft was more marked rather than in patients with bilateral cleft ($p < 0.001$). Altogether, the relative amount of apoptotic cells in the tissues in patients with bilateral cleft was lesser than in the control patients, though no statistically significant differences were observed. This finding can be explained by the severity of the cleft, and specificities of bilateral cleft-affected tissue homeostasis. In the current study we observed also a convincingly diminished cell proliferation in children with bilateral cleft lip and palate in the tissues obtained from cheiloplasty, while during soft and hard palatoplasty we could visualize an increasing cell proliferation ($p < 0.05$). The results mentioned, perhaps, depict the morphological peculiarities of the tissues of the most severe cleft type. Consequently, cell proliferation and apoptosis are of importance not only during the embryonic developmental process, but also in cleft-affected tissue homeostasis postnatally.

The theory of programmed cell death theory in the process of lip and palatal shelves development and morphogenesis has been accepted in publications of the last years, however, one can find contradictory data in the literature (Novakovic *et al.*, 2010). In the study with the mice, Cuervo and Covarrubias had proved the presence of apoptotic cells, using TUNEL method in the epithelial area of anterior palatal medial margin shortly after the palatal shelves merging, while apoptosis of posterior margin epithelial cells was noticed prior to shelves merging. Both scientists made a conclusion, that medial margin epithelium degeneration was provided by cell apoptosis, and this process was activating the basal membrane degradation during the palatal

shelves merging (Cuervo and Covarrubias, 2004). Similar results were published in a more recent study, in which Martínez-Alvarez and co-authors visualized apoptotic cells prior to palatal shelves merging (Martínez-Alvarez *et al.*, 2000). Another group of scientists from Japan, however, opposes this statement. Tahakara and the team, cultivating the mice palate and inhibiting cell death in vitro, proved that a complete absence of apoptosis does not affect the palatal merging (Takahara *et al.*, 2004). We should stress, that both before-mentioned studies were done using the experimental animals- the mice. Whereas, in a quite recently done study in Croatia (Vukojevic *et al.*, 2012) on human embryos and fetuses, there were assessed not only cell apoptoses, but also cell proliferation in the palatal shelves tissues. In this study, like in our study, there were visualized Ki-67 positive epithelial cells and apoptotic cells in cleft-affected tissues. We still cannot object, that between our study and study of Croatia there are differences in the studied tissue material. During primary palatal shelves merging Vukojevic and co-authors (2012) found proportionally decreased medial margin epithelial cell proliferation (from 42% till 32%) and cell apoptosis (from 11% till 7%) during primary palatal shelves merging, while in the epithelium of the secondary palatal shelves margin there were visualized 28% proliferating cells and 5% apoptotic cells. Cell proliferation immunohistochemically was visualized similarly to our study by using cell proliferation marker Ki-67, but cell apoptosis was identified, using TUNEL method. This study with human embryonic tissues shows, that cell proliferation and apoptosis have a significant importance not only in the development of primary and secondary palates, but, perhaps, also in the facial cleft etiopathogenesis (Vukojevic *et al.*, 2012).

Nestin and CD34 immunohistochemical profile in soft tissues of the cleft. In our study in unilateral and bilateral clefts and the control patients mucosa there was found rich amount of nestin containing epithelial cells and connective tissue cells. Comparing the nestin presence in the material, obtained

from cheiloplasty in patients with unilateral cleft and patients with bilateral cleft, there were seen significant differences. There were less positive cells in the material of bilateral clefts ($p < 0.05$). In the control patients tissue samples there was seen convincingly rich nestin presence in the oral cavity mucosa ($p < 0.001$). This finding can be explained by the decrease of tissue regeneration potential in children with clefts and more pronounced in patients with bilateral clefts.

It has been discovered, that nestin expressing cells can proliferate, differentiate and migrate. After getting the wound, cells to a greater extent express nestin, and this discovery indicates an essential role of this protein in the tissue regeneration, besides, nestin increasing expression is regulated by different growth factors and other signal molecules (Wiese *et al.*, 2004). Using different origin cell markers, among them nestin, in the recent study there was assessed the presence of oral cavity mucosal stem cells/multipotent stem cells, because oral cavity mucosal epithelial stem cells/multipotent stem cells have a significant importance in tissue homeostasis and wound healing. In this study, using the immunofluorescence method, no nestin presence was identified in the mucosa. At the same time Joas and colleagues describe nestin expression in buccal and gum mucosa (Jones and Klein, 2013). It is interesting, that nestin expression was studied also in normal and pathological human dental tissues.

In our study in all cleft cases we found rich CD34 presence in *lamina propria* blood vessel wall cells. There were more CD34 positive epithelial cells in cleft patients tissues obtained from cheiloplasty, rather than in the control patients tissues ($p < 0.001$). This finding is estimated as compensator's reaction to tissue changes, stimulating formation of new blood vessel cells.

A similar study has been done by a team of scientists from India. In this study there was assessed vasculogenesis, dealing with oral cavity mucosal fibrotic changes and epithelial atrophy, using CD34 and FGF markers. CD34 was proved to be a direct oral mucosal vasculogenesis characterizing factor. In

the pathological processes-affected oral mucosa there was found statistically significantly decreased CD34 positive structures in relation to oral mucosal atrophy (Pandiar and Shameena, 2014). A different team of scientists proved, that in the pathologically affected oral mucosa one can find an increased amount of CD34 positive structures (Desai *et al.*, 2010).

TGFβ3 and VEGF immunohistochemical profile in tissues of the cleft. In our study we found a significantly decreased relative amount of TGFβ3 positive structures in bilateral cleft lip material, in comparison to unilateral cleft and control tissue samples ($p < 0.001$; $p < 0.005$). Comparing the tissue samples obtained from osteoplasty, we observed a moderately decreased relative amount of TGFβ3 osteoblasts and osteocytes in patients with bilateral cleft and control group patients ($p < 0.001$). This fact could be explained by insufficient cell proliferation, differentiation and apoptosis in the most severe cleft type, *i.e.*, complete bilateral cleft lip and palate. Assessing the presence of this factor in repeatedly taken tissue samples, we visualized a convincingly greater amount of relative structures of TGFβ3 in soft and hard palatoplasty tissue samples ($p < 0.05$). This finding can be explained as a preserved positive tissue compensators' reaction. Besides, there was seen a moderately close positive correlation between TGFβ3 in the epithelium and connective tissue ($p < 0.05$). TGFβ3 is known to regulate cell death (Yu *et al.*, 2009). It is interesting, that in this study we found an association between TGFβ3 positive structures and apoptotic cells in children with complete unilateral cleft lip and palate ($p < 0.05$). Since TGFβ3 induces MMP-9 expression and activity (Hosokawa *et al.*, 2003), it was tested, whether between these markers there exists correlation with patients with clefts. When assessing the results, we found close correlation between TGFβ3 and MMP-9 positive connective tissue cells, and between MMP-9 positive connective tissue cells and TGFβ3 epithelial cells. By increase of one factor presence, there increases another

factor presence, which proves, that there exists close association in relation to the cleft etiopathogenesis.

TGFβ3 signal way is considered to interact with a different growth factor, transcription factor or enzyme signal ways which are involved in the cleft pathogenesis (Galloway *et al.*, 2013). For instance, Xu with co-authors has concluded, that transcription factor IRF6 is controlled by TGFβ3 (Xu *et al.*, 2006). In our study we discovered, that in children with clefts there exists the correlation between TGFβ3 and IRF6 in positive structures in the connective tissue ($p = 0.001$). The other gene protein associations with TGFβ3 were analyzed and several both close positive, and moderately positive correlations (MSX1, PAX9, RYK) were found, which in cleft-affected tissues indicate to TGFβ3 local interrelation with other factors.

Similar results to our study were acquired in Italy in 2006. In this study TGFβ3 expression was assessed in the surgical material from children with isolated palatal clefts, and lip, alveolar process and/or palatal clefts. Rullo with colleagues found in children with clefts a convincingly decreased TGFβ3 expression in the epithelium, comparing it with the control material, obtained during the tooth extraction (Rullo *et al.*, 2006). Unfortunately, TGFβ3 positivity in the connective tissues here was not assessed, while we assessed the presence of this growth factor in relation to the severity of the cleft.

Besides, TGFβ3 was identified to regulate the osteoblast differentiation, thus TGFβ3 gets involved in the bone remodelling (Bouletreau *et al.*, 2000; Chen *et al.*, 2012). During the bone resorption, osteoclasts activate latent TGFβ3, which stimulates the bone formation. This discovery shows, that TGFβ3 is the leading factor in the bone healing process phase – remodelling (Silva *et al.*, 2004). Taking into account the study results, it was concluded, that TGFβ3 is an essential, angiogenesis-influential factor in lip and palatal development just in the late developmental stages, when there takes place peridermal cell disappearance and palatal shelves merging (Muraoka *et al.*,

2005; Jalali *et al.*, 2012). TGF β 3 is known to induce ETM, by regulating cell proliferation, differentiation, ECM, and, perhaps, also MEE apoptosis (Tian and Schiemann, 2009; Galloway *et al.*, 2013). In the publications of the last years there are described the facial shelves peridermal layer cells, because their disappearance provides successful merging of lip and palatal shelves. When studying the cells of the mice palatal shelves, in the study of 2015, TGF β 3 was proved to regulate the disappearance of peridermal cells, promoting successful merging of palatal shelves (Hu *et al.*, 2015). Japanese scientists' group discovered TGF β 3 expression in the epithelial cells of the mice lip prominences, while TGF β 3 absence was seen in the mice with the cleft lip.

An interesting fact was found, that exogenously injected TGF β 3 in the mice, influences the merging of lip prominences, inducing the endothelial cell differentiation and promoting the mesenchyme cell proliferation. TGF β 3 induces the expression of two important angiogenesis promoting factors – Flk-1 and CD31 (Muraoka *et al.*, 2005). Similar conclusions were published in the study of 1998, when scientists observed, that exogenously injected TGF β 3 in hens with the cleft produces the normal merging of palatal shelves (Sun *et al.*, 1998). Ohno with colleagues injected 0.5 ml TGF β 3 in rats in the buccal submucosa and concluded, that this factor promotes epithelization, and in such a way decreases the scarring (Ohno *et al.*, 2011). The results of this study confirms the fact, that TGF β 3 promotes regeneration of oral mucosa. Assessing TGF β 3 functions in many populations, analysis was done as to genetic variations in the association of this gene to the development of the lip and palatal cleft (Saleem *et al.*, 2012; Tang *et al.*, 2013; Mehrotra, 2015).

Kohama *et al.*, in his turn, demonstrated that TGF β 3 was the leading factor in wound healing after cheiloplasty, inducing mesenchyme cell proliferation and migration towards wounded cells (Kohama *et al.*, 2002). In the experiment with the mice, it was proved, that TGF β 3 reduced the scarring *in vivo* (Hosokawa *et al.*, 2003).

Comparing complete unilateral cleft lip and palate tissue samples, acquired from patients during cheiloplasty, with those of bilateral cleft tissues, we discovered, that the average relative amount of VEGF positive structures in more severe cleft cases was decreased ($p < 0.05$). In the material acquired from hard palatoplasty, the amount of VEGF positive structures was lesser than in the control group patients ($p < 0.005$). Comparing Sundeep *et al.* study results, the finding of this work can be explained by, perhaps, impaired neovascularization and reepithelization of lip and palatal mucosal wound healing process in children with the most severe cleft type, *i.e.*, complete bilateral cleft lip and palate. However, in the tissue samples, acquired from repeated operations, we saw an increasing amount of VEGF containing structures, which gives the evidence to the late tissue ischemia, which, evidently, appears within a longer period of regeneration.

Vascular endothelial growth factor (VEGF) belongs to the family of signal molecules, which forms the blood-vessels during the time of embryonic development and wound healing, as well as ensures vascular homeostasis in the human body, and is able to inhibit apoptosis (Roy *et al.*, 2006; Andisheh-Tadbir *et al.*, 2014). The studies discovered, that VEGF and its receptor VEGFR-2 express themselves in a healthy human's epiderma, hair follicule external epithelial sheath, regulating epitheliocyte proliferation and migration, as well as decreasing the adhesion capacity of these cells (Man *et al.*, 2006; Li *et al.*, 2012). There are no exact data in the literature on the importance of this factor's involvement in the facial cleft pathogenesis. For instance, Patyna *et al.* in his study, treating rats and rabbits with VEGF inhibitor SUTENT® ≥ 1 mg/kg/daily described the development of lip and palatal clefts in rabbits (Patyna *et al.*, 2009). In a different study, in its turn, there was proved, that TBX1 protein, being important in the development of facial and cervical blood-vessels, probably plays the role in small blood-vessel pathology in embryonic tissues. Microdeletions in certain loci of this gene produce DiGeorge

Syndrome, which clinically is characteristic for the palatal cleft. Reduced amount of VEGF decreases TBX1 function, therefore there may develop vascular malformations (Stalmans *et al.*, 2003). Vascular malformations in the mesenchyme of lip and palatal shelves, perhaps, negatively affect the tissue remodelling, therefore the expression of other signal molecules and tissue merging are changed (François-Fiquet *et al.*, 2014).

PGP 9.5 immunohistochemical profile in soft tissues of the cleft. In our study PGP 9.5 containing nerve fibres were observed in all unilateral and bilateral cleft cases. PGP 9.5 contained single nerve fibres in the epithelium and subepithelium, as well as nerve fibres around the sebaceous glands, hair follicles and blood vessels. Small nerve fibres were visualized closer to the basal membrane. Also in the blood vessel walls we observed PGP 9.5 positive nerve fibres. In some samples in the epithelium – *stratum basale* and *stratum spinosum* layers there were found PGP 9.5 containing neuroendocrine cells. Our study results were similar, although they did not agree with the study results, which had been acquired by scientists from Brasil. The latter were assessing the lip, buccal and palatal mucal innervation in rats, using the immunohistochemical method and transmission electron microscopy analysis. Similarly to our study, Watanabe *et al.* also found a lot of PGP 9.5 positive nerve fibres in rats oral mucosal connective tissue (Watanabe *et al.*, 2013), although, in total, quite few to moderately enough amount of (+/++) positive nerve fibres were found. Similar, though much earlier carried out study, was done in 2000, analyzing the rats hard palate mucosal innervation with PGP 9.5. As a result, it was concluded, that the hard palatal mucosa was well innervated (Mitsui *et al.*, 2000). This finding, perhaps, has to be related to the differences in the material studied.

In the material of bilateral cleft-affected tissues, we saw PGP 9.5 distribution differences, i.e., the relative amount of PGP 9.5 containing structures in the bilateral cleft material was decreased, in comparison to the

tissue samples from patients with unilateral cleft ($p < 0.05$), which, perhaps, was due to the cleft severity. Besides, we observed, that in the tissues acquired during soft palatoplasty, positive structures of PGP 9.5 were lesser than in the tissues acquired from lip and hard palate plasty ($p < 0.05$). It may be, that soft palate mucosa in cleft patients is lesser innervated. Kato *et al.* in the study with animal models had concluded, that hard and soft palate transition zone is well innervated (Kato *et al.*, 1998), besides, the study results of a different authors team show, that not only the transition zone, but the area of the soft palate is sufficiently well innervated (Shimokawa *et al.*, 2005). These results may, perhaps, be associated with the differences of species.

MMP and TIMP immunohistochemical profile in the cleft-affected hard tissue. Assessing MMP-2 and TIMP-2 presence in the material acquired in osteoplasty, there were observed decreased this protein containing structures in children with the most severe cleft type, *i.e.*, complete bilateral cleft lip and palate ($p < 0.001$). This finding, perhaps, can be explained by the fact, that the alveolar bone remodelling potential in patients with bilateral cleft is less marked than in patients with unilateral cleft.

Molecular mechanisms of alveolar bone remodelling in relation to facial clefts and osteoplasty used for their treatment have not been widely studied. In a recent study MMP-2 expression was proved, as well as its role in the alveolar bone regeneration in rats after a tooth extraction (Accorsi-Mendonça *et al.*, 2008). Scientists from Brasil reported, that by stimulating MMP-2 expression, there is stimulated the bone remodelling and successful wound healing (Rocha *et al.*, 2014). It was found as well, that a decreased MMP-2 functional activity was negatively influencing the bone quality (Nyman *et al.*, 2011). Kramer *et al.* investigated, that by injecting TIMP-2 into the cranial bone defect areas, there was stimulated the healing process (Kramer *et al.*, 2008).

During osteoplasty the alveolar bone cleft is being corrected with autotransplanted osteocytes from tibia or crista iliaca superior area, which, at

present, is considered the golden standard in the treatment of alveolar bone defect in the complete cleft at the age of bite exchange (Herford *et al.*, 2007). The main aim of osteoplasty is to fill in the defect site, to stabilize maxilla, to close the oronasal fistula, to ensure the teeth eruption and to improve the nasal symmetry (Bajaj *et al.*, 2003). Considering the fact, that the alveolar bone healing and remodelling are a complex process, coordinated by the cells, bioactive factors and ECM, stimulating preosteoblast proliferation, differentiation and migration, there are investigated the abilities of different factors to affect the bone regeneration.

BMP2/4 immunohistochemical profile in cleft-affected hard tissue.

In our study we observed significantly decreased BMP2/4 presence in children with bilateral cleft ($p < 0.001$). Altogether in children with complete unilateral cleft lip and palate there was visualized rich BMP2/4 presence in osteocytes. Thus, bone healing and regeneration capacities in patients with unilateral clefts even after the bone trauma due to the operation were more pronounced than in patients with the most severe cleft type, as well as in more severe cleft cases the bone healing and growth potential were essentially reduced. The finding in the control patient's material give evidence to the importance of BMP2/4 in the regulation of normal tissue function. The discovery made in this study shows, that children with bilateral cleft have a reduced bone regeneration potential, therefore there may be a greater probability, that the wound in these children would heal with a permanent defect. Similar results were acquired by Alarmo and Torrecillas-Martínez *et al.* (Alarmo *et al.*, 2013; Torrecillas-Martínez *et al.*, 2015).

In recent studies with animal models it was proved, that can also BMP-4 induce the hard tissue regeneration (Shi *et al.*, 2013; Shiozaki *et al.*, 2013). Torrecillas-Martínez in his study emphasizes, that BMP-4 plays an essential role in the bone homeostasis and regeneration after surgical expansion of maxillary sinus (Torrecillas-Martínez *et al.*, 2015). One can find also some

recent studies, pointing to BMP-4 gene positive association with the lip with/without palatal clefts in different populations (Kempa *et al.*, 2014; Chen *et al.*, 2014; Hu *et al.*, 2015). Suzuki *et al.*, using the immunohistochemical method, identified BMP-2 expression in dental germs and mandibular tissues in the human embryos, which proved the importance of this protein in morphogenesis of facial and oral tissues (Suzuki *et al.*, 2001).

The basic substance of non-collagen of alveolar bone and OPG immunohistochemical profile. Altogether OC containing cells in patients with unilateral and bilateral alveolar bone cleft were found in all tissue samples. Both in patients with the cleft, and in the control group patients the finding was similar, though in the control group patients the relative amount ($p < 0.05$) of OC containing structures was statistically significantly higher. When assessing this finding, we concluded, that in patients with the cleft the alveolar bone mineralization was proceeding actively.

OC importance is proved in the bone mineralization, bone formation and resorption process (Murshed *et al.*, 2004). Already since 1996, the studies with the mice have proved, that the lack of OC increases the osteocyte mass and affects its quality (Ducy *et al.*, 1996). It was also found, that OC stimulates angiogenesis, which strengthens its importance even to a greater extent in the bone healing and remodelling (Cantatore *et al.*, 2005). OC expression was assessed during the period of alveolar bone healing in the mice, during which there was seen its increasing expression in the period, when an intensive bone mineralization was taking place (Rodrigues Luvizuto *et al.*, 2010).

We assessed OPN presence just in the tissues, obtained from the area of alveolar bone cleft, and identified a significantly reduced OPN presence in the control group patients and children with the cleft of bilateral alveolar process, in comparison to patients who had unilateral cleft ($p < 0.001$; $p < 0.003$). We found out, that these results, firstly, show the degree of cleft severity. Secondly, assessing the function of this factor in the tissues, OPN probably decreases cell

mobilization, migration, resistance to apoptosis, as well as prevent angiogenesis during alveolar bone healing and remodelling.

OPN is a potential palatal and alveolar bone contributing factor (Jono *et al.*, 2000). Besides, in the two recent studies OPN role in the mice palate development was emphasized, and significant OPN expression changes were found within the period of craniofacial development (Mukhopadhyay *et al.*, 2004). In their study Jakobsen *et al.* published interesting results about OPN, or significance of secretory phosphoprotein in the development of human palate, as well as the lip and palatal clefts in the possible etiopathogenesis. In this study using Gene Chip and RT-PCR test, there was found a statistically significantly increased OPN expression in the palatal material of patients with lip and palatal cleft (Jakobsen *et al.*, 2009). Using the animals in the experiments, it was revealed that a changed OPN expression prevents early vascularization, changes ECM organization, and bone remodelling (Duvall *et al.*, 2008).

OPG in this study was assessed as an effective bone remodelling inhibiting factor. In total, OPG presence in children with bilateral alveolar bone cleft was convincingly lesser, in comparison to patients with unilateral cleft, and the control group patients ($p < 0.001$; $p < 0.05$).

The studies with the mice revealed, that, as a result of local OPG insufficiency, the bone modelling (Dunn *et al.*, 2007; Ma *et al.*, 2008) is inhibited. In vivo study OPG gene therapy was found to protect the alveolar bone from excessive resorption, induced by periodontitis (Tang *et al.*, 2015). Estimating the results of the work done, OPG finding shows, that children with bilateral alveolar process cleft have a decreased bone protection, which can negatively affect the postoperative regeneration process of the alveolar bone.

Analyzing the correlations of the acquired result, it was found, that there exists statistically significant correlation between OC, OP and OPG osteocytes,

acquired from patients with unilateral cleft. It means, that with the increase of OC expression, there increases also OP and OPG expression, and by the increase of OP expression, there increases OPG expression. It identifies to the balanced alveolar bone remodelling process. In patients with the most severe clefts, i.e., bilateral alveolar bone cleft, this correlation is weaker pronounced.

At the end of the discussion, we have to say, that for the first time in the current study, there was holistically analyzed the most severe type of clefts – complete bilateral cleft lip and palate tissue morphological changes in the ontogenetical aspect. Assessing MMP and TIMP presence in complete cleft lip and palate-affected tissues, it was clearly seen, that from tissue degeneration factors in bilateral cleft patients tissues there was dominating MMP-2, but as an inhibiting factor – TIMP-2. It is interesting, that in patients with unilateral cleft MMP-9 and TIMP-4 dominating presence were convincingly visualized. Since it has been proved, that a heightened level of MMP-9 reduced the scarring, it was concluded, that for patients with bilateral cleft there may be a greater probability, that the operation induced mucosal healing phases will be negatively affected, and they would heal with an increased risk of the scarring. MMP-2 role in the process of scarring is still unclear. In children with bilateral cleft in soft and hard palatal tissues there was found increased MMP-9 and TIMP-4 presence, but MMP-2 and MMP-8 presence was reduced. There were also found significant correlations between MMP and TIMP positive cells both in the epithelium, and in the connective tissues. The identified correlations show the mutual relationship of MMP and TIMP, i.e., if one factors function is impaired, there is influenced a different enzymes functional activity as well.

Altogether, in the most severe cleft type, i.e., complete bilateral cleft lip and palate, there was found the transcription factor IRF6, MSX1 and PAX9, the growth factor TGF β 3 and VEGF, cell proliferation and apoptosis decreased presence in the soft tissues, but in supporting tissues the decreased OPN, OPG,

MMP-2, TIMP-2, BMP2/4 and TGF β 3 presence. We convincingly identified, that morphological tissue changes are more severe in patients with bilateral cleft. During repeated operations in one and the same patients we observed increased MSX1, MMP-9, TIMP-4, TGF β 3, Ki-67 and decreased VEGF expression.

4. CONCLUSIONS

1. The most characteristic, nonspecific changes in bilateral cleft lip and palate patient tissues are: oral mucosal epithelium vacuolisation and/or basal layer cell hyperplasia, formation of epithelial network protuberance, as well as connective tissue fibrosis and pronounced neovascularization in *lamina propria*, but in the osteocytes – irregular osteon arrangement in the bone with a focal connective tissue proliferation.

2. In the cheiloplasty-obtained material in complete bilateral cleft lip and palate patients one can see pronounced degeneration in the enzyme MMP-2 presence and its inhibitor TIMP-2 presence, while MMP-8 and MMP-9, and MMP-4 expression dominate in unilateral cleft material. Increased MMP-9 expression proves the postoperative tissue scarring without any significantly permanent scarring in children with complete unilateral cleft. In complete bilateral cleft-affected tissues the degeneration enzyme MMP-8 expression decreases, and its inhibitor TIMP-4 expression increases, with the increase of the child's age.

3. In complete bilateral cleft lip and palate patients the little MSX1, IRF6 and PAX9 expression in oral tissues is related to decreased cell proliferation, differentiation, migration, restoration, as well as programmed cell death prevention, determining a severe cleft type. But the increasing IRF6, PAX9 expression determines the functional activity of cell proliferation, differentiation in cleft-affected soft and hard palatal tissues. Essential statistically significant correlation between PAX9 and IRF6, as well as between PAX9 and MSX1, IRF6 and RYK expression in soft tissues indicate to morphogenetical mutual gene effect in cleft cases.

4. Complete bilateral cleft-affected soft tissues are characterized by markedly poor TGFβ3 presence, which indicates insufficient cell migration,

proliferation, differentiation and apoptosis in the more severe cleft type, while the osteocytes are characterized by statistically more pronounced expression of this factor, identifying the compensatory adjacent tissue reaction and essential significance of the factor in certain cleft type pathogenesis.

5. Bilateral cleft-affected soft tissues are characterized by decreased VEGF presence, which indicates to the initial lack of ischaemia in the tissues and, perhaps, is connected with a changed neovascularization and reepithelization. Unilateral and bilateral cleft lip and palate-affected soft tissues are characterized by pronounced CD34 expression, indicating to the local potential of increased vascular cell formation.

6. In complete bilateral cleft lip and palate cases the statistically decreased nestin containing cell presence in the tissues obtained during cheiloplasty substantiate the limitation of neural cell differentiation.

7. The decrease of relative amount of apoptotic cells in bilateral cleft patients identifies the decrease of soft tissue remodelling in a more severe cleft type.

8. The decrease of relative amount of statistically significant BMP2/4, TGFβ3, MMP-2, TIMP-2 containing structures in complete bilateral cleft lip and palate patients identifies the decrease of the alveolar bone regeneration and remodelling process.

9. The statistically significant decrease of OPG and OPN in bilateral alveolar process of cleft patients identifies the osteocyte cell functional activities, at the same time – the decrease of cell proliferation potential and bone resorption increase in a more severe cleft type, but at practically unchanged mineralization background (OC).

10. Complete bilateral cleft lip and palate-affected tissues have a characteristically increasing MSX1, MMP-9, TIMP-4, TGFβ3, Ki-67 and

decreased VEGF expression in ontogenesis, which indicates to the dominating role of these factors in regulating cleft-affected tissue compensatory abilities after repeated operations.

11. Bilateral cleft tissues and, especially, soft palate, are characterized by decreased neuropeptide containing innervation. This specificity characterizes the degree of the disease severity, which develops, most probably, due to the lack of tissues, and also due to the deficit of nestine containing neural precursors.

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In Latvian language

1. Akota I., Barkāne B., Grasmane N. Iedzimto sejas šķeltnu biežuma rādītāji Latvijā no 1960. līdz 1997. gadam. // *AML/RSU Zinātniskie raksti 2000.* – Rīga: AML/RSU, 2001, 166.–170. lpp.
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6. LIST OF PUBLICATIONS

Scientific papers (5)

1. **Smane L.**, Pilmane M., Akota I. Local expression of inflammatory cytokines in the facial tissue of children with a cleft lip and palate. *Papers on Anthropology XXI*. 2012; 264-275.
2. **Smane L.**, Pilmane M., Akota I. Apoptosis and MMP-2, TIMP-2 expression in cleft lip and palate. *Stomatologija*. 2013; 15(4): 129–134.
3. **Smane L.**, Pilmane M. IRF6, RYK, and PAX9 expression in facial tissue of children with cleft palate. *Int. J. Morphol.* 2015; 33(2): 647–652.
4. **Smane L.**, Pilmane M. Osteopontin, osteocalcin, and osteoprotegerin expression in human cleft lip and palate affected tissue. *SHS Web of Conferences* 30. 2016: 1-9. doi: 10.1051/shsconf/20163000008.
5. **Smane-Filipova L.**, Pilmane M., Akota I. MMPs and TIMPs expression in facial tissue of children with cleft lip and palate – accepted for publication in *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* 2016; 160: XX.

Abstracts and presentations in international conferences (13)

1. **Smane L.**, Pilmane M., Akota I. Local expression of inflammatory cytokines in facial tissue of children with clefts lip and palate, 11. Joint Symposium der Universitäten Riga und Rostock abstract, Rostock 2012, abstract published in homepage of the symposium. (Oral presentation).
2. **Smane L.**, Pilmane M., Akota I. Immunohistochemical detection of growth factors, protein gene product 9.5 (PGP 9.5) in cleft lip and palate, Baltic

- Morphology 7th scientific conference abstract, Riga 2013, abstract book 51. p. (Best Poster Award – First Place).
3. **Smāne L.**, Pilmane M., Akota I. Analysis of apoptosis and expression of specific signaling proteins in facial tissue of children with clefts lip and palate, 21st International Conference on Oral and Maxillofacial Surgery abstract, Barcelona 2013, abstract book 183. p. (Poster presentation).
 4. **Smāne L.**, Pilmane M., Akota I. IRF6, RYK and PAX9 role in facial tissue of children with clefts, 18th Congress of the International Federation of Associations of Anatomists abstract, Beijing 2013, *Annals of Anatomy* 2014; 196(S1): 86. (Poster presentation).
 5. **Smāne L.**, Pilmane M., Akota I. Immunohistochemical analysis of nestin, CD34 and TGFβ3 in cleft lip and palate, XXIV European Congress of Perinatal Medicine abstract, Florence 2014, *J Matern Fetal Neonatal Med.* 2014; 27(1): 275. (Poster presentation).
 6. Pilmane M., **Smāne L.**, Akota I. Degeneration and remodeling potential in hyaline cartilage and bone of complete cleft lip palate affected facial tissue in children, International Conference REDEOR abstract, Venice 2015. (Poster presentation).
 7. **Smāne L.**, Pilmane M., Akota I. Expression of transcription factors MSX1 and PAX9 in a group of Latvian patients with complete unilateral and bilateral cleft lip and palate, Baltic Paediatric Congress abstract, Riga 2015, abstract book 70. p. (Poster presentation).
 8. **Smāne L.**, Pilmane M., Akota I. Association of gene proteins with cleft lip and palate in the Latvian population, 2nd World Congress on Controversies in Pediatrics abstract, Budapest 2015, abstract book 64. p. (Poster presentation).
 9. **Smāne L.**, Pilmane M., Akota I. Osteopontin, osteocalcin, osteoprotegerin expression in facial tissue of children with cleft lip and palate, 5th

- International Interdisciplinary Scientific Conference “SOCIETY. HEALTH. WELFARE” abstract, Riga 2015, abstract book 68. p. (Poster presentation).
10. **Smāne L.**, Pilmane M., Akota I. Expression of non-collagenous proteins in facial tissue of children with cleft lip and palate, Experimental Biology abstract, Boston 2015, FASEB J. 2015; 29: 765.2. (Poster presentation).
 11. **Smāne L.**, Pilmane M., Akota I. Patterns of MMP-2, TIMP-2, BMP2/4, and TGFβ3 expression in alveolar bone tissue of children with orofacial clefts, Baltic Morphology 8th scientific conference abstract, Vilnius 2015, abstract book 65. p. (Best PhD Student Oral Presentation Award).
 12. **Smāne-Filipova L.**, Pilmane M. “Functional morphology of tissues in children with complete bilateral cleft lip and palate” 6th International Congress of UENPS abstract, Valencia 2016. (Poster presentation).
 13. **Smāne-Filipova L.**, Pilmane M., Akota I. “Evaluation of VEGF and CD34 protein expression in facial tissue of children with cleft” 6th International Interdisciplinary Scientific Conference “SOCIETY. HEALTH. WELFARE” abstract, Riga 2016. (Poster presentation).

Abstracts and presentations in local conferences in Latvia (7)

1. **Smāne L.**, Pilmane M., Akota I. Iekaisuma signālmolekulu izpēte bērniem ar lūpu un aukslēju šķeltnēm, annual Rīga Stradiņš University Scientific Conference 2012 abstract, abstract book 316. p. (Poster presentation).
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