RĪGA STRADIŅŠ UNIVERSITY

IVETA JANKOVSKA

Characterization of regeneration/growth factors, degeneration factors and apoptosis in jaw bones and soft tissues of patients with class II and class III dentofacial deformities

(speciality – orthodontics)

Summary of promotion work

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Promotion work was carried out by support of ESF project „Support of doctoral students on acquiring the doctoral study programme and obtaining a scientific degree at Rīga Stradiņš University”

Rīga, 2011

Promotion work was carried out at:

Rīga Stradiņš University Institute of Anatomy and Anthropology
Rīga Stradiņš University Institute of Stomatology

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Defence of promotion work will be held 23rd February 2011, 5.00 pm at the meeting of Promotion council of Stomatological disciplines of Riga Stradiņš University at RSU Hippocrates lecture room in Riga, Dzirciema street 16.

The promotion work is available at RSU library

Secretary of Promotion council:

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TOPICALITY OF STUDY

Dentofacial anomalies of a rather great severity in Latvia's population at the age of 18 years are encountered in 5 – 7% cases (Urtāne, 2006) and cause functional, esthetical, as well as psychological disorders. Their formation is connected with atypical jaw growth and tissue remodelling disorders, which may be genetically determined and/or due to post-natal developmental factors, resulting in jaw hypoplasia or hyperplasia which are at the basis of skeletal dentofacial anomalies. The bone as a form and size of an organ is constantly developing during the bone tissue remodelling process, where at the cellular level the lines of osteoclast and osteoblast cells are functioning in coordination. The changes of activities of these cell lines lead to deviations from the accepted norm of the bone form and size. At the level of molecular biology the cell activity is induced and maintained by the growth factors. In the bone tissue structure an important part lies in the content of intercellular substance or extracellular matrix, its formation and degradation. The cell death is as important in physiological conditions cells end their life-cycle in the way of apoptosis.

The management of dentofacial anomalies is combined and interdisciplinary. It consists of orthodontic treatment using a fixed bracket system and orthognatic surgery. Orthodontic treatment is usually undertaken at the age of 17 – 18 years, when the intensive jaw bone growth has stopped and the results of planning for their position is predictable. The patient is prepared for the orthognatic surgery, performing the aligning of teeth rows and shifting them to a such position which would correspond to the preferable jaw proportions. During the orthognatic surgery the jaw bone osteotomy is performed by shifting of fragments, fixation into correct skeletal and dental proportions which is followed by longstanding consolidation and remodelling process. However, the combined treatment, based on modern orthognatic surgery planning, orthodontic and surgical technology methods does not exclude the cases of corrected tissue relapse as a result of bone tissue remodelling, which requires repeated treatment causing quite a great extra expenses. Depending on the type of the osteotomy done, the relapse is seen in 10 – 30 % patients (Mobarak, 2000).

Most commonly the skeletal morphology of dentofacial deformation deals with jaw hypoplasia or hyperplasia, which correspond to class II or class III malocclusion. Clinically the jaw bone deformation manifestations are similar, however, by X-ray cephalometry data these anomalies were found to have an individually different skeletal morphology, which gives proof to the manysided etiopathogenseses of these anomalies. Thus, the determination of the growth factors, bone extracellular matrix proteins, degeneration enzymes, gene proteins

and apoptosis in the jaw bones in patients with dentofacial anomalies would provide an extra information on the pathogensis of anomalies and patients' individual bone structure morphogenesis.

AIM OF STUDY

The aim of the promotion work was to study the factors which are important for bone remodelling process, such as the growth factors, extracellular matrix proteins, degeneration enzyme, gene proteins and apoptosis specificities in jaw bone growth zones in class II and class III patients with dentofacial deformaties and compare them to a control group.

OBJECTIVES OF STUDY

1. Using histological staining with hematoxiline and eosine, to determine the histological bone tissue changes in skeletal class II and class III patients.
2. Using immunohistochemical method, to determine the expression of the growth factor and its receptors (transformating growth factor β (TGF-β), the bone morphogenetic protein 2/4 (BMP2/4), fibroblast growth factor receptor 1 (FGFR1) and vascular endothelial growth factor (VEGF)) in bone tissue and the surrounding soft tissue samples in patients with with skeletal class II and class III malocclusion.
3. Using immunohistochemical method, in bone tissues and soft tissues to determine the expression of bone extracellular matrix proteins (osteopontin (OP) and osteocalcin (OC)) and extracellular matrix degeneration enzyme (matrix metaloproteinase 2 (MMP2)) in patients with class II and class III dentofacial deformaties.
4. Using immunohistochemical method, to determine the gene proteins (barx1, msx2 and wnt1) expression in patients with class II and class III dentofacial deformations.
5. Using TUNEL method, to determine the parameters of apoptosis in tissue samples in patients with class II and class III dentofacial deformaties.
6. To state the correlation between the growth factors, bone extracellular matrix proteins, degeneration enzyme, gene proteins and apoptosis expression and types of dentofacial deformaties.
7. To compare the acquired data to the findings of the control group patients.

HYPOTHESIS OF STUDY

The parameters important for bone tissue morphogenesis, such as growth factors and their receptors (TGF-β, BMP2/4, FGFR1 and VEGF), bone extracellular matrix proteins (OC, OP),

degeneration enzyme (MMP2), gene proteins (barx1, msx2, wnt1) and apoptosis are different in jaw bone growth zones in various dentofacial deformation groups.

NOVELTY OF STUDY

1. A new information is acquired on the presence of the growth factors, bone extracellular matrix proteins, degeneration enzyme, gene proteins and apoptosis in jaw bone growth zones in patients with class II and class III dentofacial deformations and the control group patients.

2. There are determined the differences of the growth factors, bone extracellular matrix proteins, degeneration enzyme, gene protein expression and apoptosis, i.e., the indices of bone growth and remodelling, in different jaw bone deformation groups, as well as in the control group patients in various jaw bone growth zones.

THE STUDY STRUCTURE AND VOLUME

The promotion work is written in the Latvian language. It consists of 13 chapters: introduction, literature review, material and methods, results, discussion, summary conclusions, list of literature, list of publications on the research theme and the appendix. Introduction deals with the aim and objectives of the study, its topicality and the hypothesis of the theme. Total size comprises 136 pages, including 53 tables and 24 pictures. In the appendix there are 42 microphotographic pictures. The list of literature includes 226 titles of the articles used.

MATERIAL AND METHODS

Study group

In the study groups were included 20 skeletal class II patients and 20 skeletal class III patients, who had been diagnosed dentofacial deformations and who needed a combined orthodontic and orthognatic surgery treatment. The patients' mean age was 20,64 ± 3,27 years and after independent selection t-test male and female mean ages did not statistically significantly differ ($t = 0,520$; $p = 0,606$). In class II group there were 13 women and 7 men, but in class III group - 14 women and 6 men.

Patients with severe general diseases in the operation time and in the anamnesis, patients with lip and/or palatal cleft, dentofacial syndromes and skeletal asymmetries were not included in the study.

In Rīga Stradiņš University Institute of Stomatology Department of Maxillofacial Surgery biopsy samples were taken during orthognatic surgery from patients' jaw osteotomy sites in the upper jaw - *tuber maxillae,* in the lower jaw - *ramus mandibulae* anterior and posterior part, as well as from the lower jaw gingival transitory fold in the second molar region.

Tissue material, just after being obtained from the osteotmy site, was fixed in Stefanini solution and forwarded to the Institute of Anatomy and Anthropology for its further processing.

From the jaw tissue samples there were made cuts which were stained by hematoxiline and eosine for light microscopy. Samples were stained with standard immunohistochemistry to detect TGF-β, BMP2/4, FGFR1, VEGF, OC, OP, MMP2, barx1, msx2 and wnt1 expression. Using TUNEL method the apoptotic cells were determined in the tissue material.

Tissue samples for the study were used in conformity with the permission of the Ethics Committee of Rīga Stradiņš University (the decision was adopted 09.11.2006).

Control group

5 patients who underwent the extractions of impacted third molars in the upper and lower jaw at Rīga Stradiņš University Institute of Stomatology Department of Maxillofacial Surgery were included into a control group.

In the control group were included patients with skeletal and dentoalveolar class I, aligned teeth rows with no teeth and teeth row anomalies, the patients' age was within 17 to 21 years, who had not had any orthodontic treatment, no severe general illnesses were seen during extraction and anamnesis, lip and/or palatal cleft or dentofacial syndromes, as well as there were not observed any clinical signs of inflammations or pain prior to extraction.

The patients' mean age was 19,4 ± 2,7 years, from them 3 were women but 2 - men. During the extraction of the third molar the tissue samples were taken from *tuber maxillae* and *ramus mandibulae* anterior part, but, considering the extraction method, and in order not to cause extra tissue damage to the patient, there were not obtained tissue samples from *ramus mandibulae* posterior part.

X-ray examinations of study group

For the assessment of facial and dentoskeletal proportions, prior to the treatment there was done a digital lateral cephalometry test at Rigas Stradiņš University Institute of Stomatology by *Trophy, Trophypan C* digital X-ray apparatus. It is an X-ray picture in a lateral projection, when a patient is placed in a standard position – looking into distance with

a natural position of the head. Assessment of pictures was done by means of cephalometric analysis programme *Dolphyn Imaging 10.5*. The following anatomical points were marked– *Sella* (S), *Nasion* (N), *A* (A), *B* (B), *Anterior nasal spine* (ANS), *Posterior nasal spine* (PNS), *Condylus* (Co), *Gonion* (Go), *Gnathion* (Gn), and 3 angular measurements (SNA, SNB, ANB) and 5 linear measurements (ANS-PNS, Co-Go, Co-Gn, AFH, PFH) were analyzed and the ratio between the posterior facial height (PFH) and anterior facial height were calculated (AFH), which was expressed in % (PFH:AFH).

Methods and reagents

In order to get a view, a routine histology method was used and the samples were stained with hematoxiline and eosine (Лилли, 1969) and microscopically analyzed by Leic BME microscope.

Biotine and streptovidine immunohistochemical method was used for the detection of expression of the growth factors and its receptors, bone extracellular matrix proteins, extracellular matrix degeneration enzymes and gene proteins (Table 1).

Table 1. Information on growth factors, their receptors, bone extracellular matrix proteins, extracellular matrix degeneration markers and gene proteins determined by biotin-streptavidine immunohistochemical method.

Factor	**Source**	**Code**	**Dilution of material**	**Manufacturer and country**
TGF-β	Mouse	1279	1: 1000	*Cambridge Science Park*, UK
BMP2/4	Goat	av1024011	1:100	*RD Systems*, UK
FGFR1	Rabbit	ab10646	1:100	*Abcam*, UK
VEGF	Mouse	M7273	1:50	*Dako*, Dānija
MMP2	Goat	AF902	1:50	*RD Systems*, UK
OC	Mouse	ab 13418	1:100	*Abcam*, UK
OP	Rabbit	ab 8448	1:100	*Abcam*, UK
barx1	Rabbit	ab 26156	1:250	*Abcam*, UK
msx2	Mouse	ab 22601	1:400	*Abcam*, UK
wnt1	Rabbit	ab15251	1:100	*Abcam*, UK

For recording of the relative frequency of indices detected by the immunohistochemical method there was used a semi-quantitative counting method widely used in the literature (Tobin *et al*, 1990; Pilmane, 1997; Table 2). Expression frequency of factors was analyzed in three visual fields of one cut. In order to process the data statistically, the number of cells observed in the microscope visual field, was coded (0 – none of positive structures were seen

in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

Table 2. Designation of relative frequency of semi-quantitative method of immunohistochemically determined growth factors, bone extracellular matrix factors, bone extracellular matrix protein, degeneration enzymes and gene proteins.

Designations used	**Explanations**
0	None of positive structures were seen in the visual field
0/+	Rare occurance of positive structures in the visual field
+	Few positive structures in the visual field
+/++	Few to average positive structures in the visual field
++	Average to many positive structures in the visual field
++/+++	Averagely many to many positive structures in the visual field
+++	A lot of positive structures in the visual field

To determine the relative frequency of apoptosis the TUNEL method (Negoescu *et al.*, 1998) was used.

Data statistical processing methods

The aim of the data statistical processing was to evaluate various cell expressions in patient groups and, by means of statistical methods, to check the validity of acceptance or denial of hypotheses advanced in the work.

The number of cells observed in the microscope visual field were coded and entered into MS Excel table, which afterwards was converted into statistical batch PASW (SPSS Inc., USA) Statistics 18 version data table. All calculations were done also in this programme.

For describing the study and control groups there were used descriptive statistic methods (Altman, 2000). Depending on the type of the variable there were calculated central tendency indices (mean arithmetic) and dispersion indices (standard deviation and standard error).

For hypotheses test there were used adequate parametric (t test and dispersion analysis (ANOVA)) and non-parametric (Kraskel-Walis and Manna-Witney) methods. For zero hypothesis denial and acceptance of alternative hypothesis in all cases significance level $p \leq 0,05$ was used.

In cases when one could not use the parametric data statistical processing methods, there were used non-parametric methods. For the comparison of two or several variables, there were used the correlation analysis methods. Correlation coefficient, as a quantitave parameter of coherence closeness between two or several variables, was calculated by range scale values – Spearman range correlation coefficient, but with measured values – Pearson correlation coefficient (Christensen, 1996; Teibe, 2001). Qualitative correlation bettween the variables in the study, on the basis of correlation coefficient r, was evaluated as weak, moderate or firm. Like criteria the following values were used: if r = 0 - 0,4, then the correlation is stated as weak, if r = 0,4 - 0,7, then the correlation is stated as moderately firm, but if r reaches 0,7 - 0,9, then the correlation is stated as firm.

RESULTS

General characteristics of cephalometric measurements

Analyzing the mean indices in patients with skeletal and dentoalveolar class II, we observed a decreased SNB angle (74,4°), increased ANB angle (6,5°), but norm-corresponding SNA angle, which, in total, shows to lower jaw retrognaty.

Table 3. Information on class II patients' measurements of cephalometric parameters (mean value (mean), standard deviation (SD), minimal value (Min), maximal value (Max)).

Measurements	**Mean**	**SD**	**Min**	**Max**
SNA (º)	81,3	4,2	74,1	89,6
SNB (º)	74,4	3,3	70	82,7
ANB (º)	6,5	2,1	3,7	9,7
PFH (mm)	78,4	8,9	62,2	93,8
AFH (mm)	111,5	7,2	97,5	128,8
PFH:AFH (%)	70,4	6,9	56,9	81,2
ANS-PNS (mm)	53,2	4,3	45	59,9
Co-Gn (mm)	111	8,5	96,7	124,8
Co-Go (mm)	58,7	7,5	47,2	69,8

Mean value between the facial posterior height and anterior height, which is expressed in % (PFH:AFH), was increased, showing a tendency to the horizontal facial growth type, while in the patient group there were also patients with a marked vertical growth, which is seen by the

minimal value of the measurement (56,9 %) and patients with a marked horizontal growth (81,2 %). Skeletal class II patients were seen to have also a diminished facial anterior height and increased facial posterior height which is related to PFH:AFH and point to the horizontal growth type (see Table 3).

Patients with skeletal and dentoalveolar class III malocclusion were seen to have increased SNB angle mean index and ANB angle mean value was -3,8°, but SNA angle mean index corresponded to the norm, indicating to prognatic lower jaw position. However, there were patients with a decreased SNA angle (74,1°), which corresponds to the upper jaw retrognaty. PFH:AFH mean value was 65,5 %, indicating to the norm-corresponding growth type, but when analyzing minimal and maximal values of the measurements, we can see, that in the group there are patients with a marked vertical (55,5 %), and horizontal (73,2 %) facial growth type (see Table 4).

Skeletal class III patients were seen to have a very great diffeerence between minimal (99,8) and maximal (140,3) facial anterior height value, pointing to the fact that in the study were included patients with a markedly different growth type. There were not so great differences between minimal and maximal posterior facial height value.

Table 4. Information on skeletal class III patients' cephalometric measurements (mean value (mean**), s**tandard deviation (SD), minimal value (Min), maximal value (Max)).

Measurements	**Mean**	**SD**	**Min**	**Max**
SNA (º)	80,7	3,3	74,1	87,8
SNB (º)	84,6	3,6	78,1	91,3
ANB (º)	-3,8	3,14	1,5	-11
PFH (mm)	79,4	6,4	72,1	95
AFH (mm)	121,6	11	99,8	140,3
PFH:AFH (%)	65,5	5	55,5	73,2
ANS-PNS (mm)	52,8	3,5	47,5	59,3
Co-Gn (mm)	129,8	9	109,9	147,1
Co-Go (mm)	62,8	4,8	56,9	74,4

Morphologic findings in bone tissue and mucosa

Analyzing the reviewed specimens in routine histologic staining with hematoxilin and eosine, in soft tissue material from lower jaw gingival transitory fold in the second molar region both in class II and class III patients group were seen the following changes: In the superior layers of epithelium were found uneven vacuolization of polimorphous epiteliocytes and basal cell hyperplasia, but in connective tissues were seen to have inflammatory cell infiltration. In some specimens uneven thickened basal membrane was found.

Analyzing control or class I group patients' histologic preparations of mucosa, which are stained with hematoxilin and eosine, in light microscopy we observed the structure of gingival mucosa, which corresponded to the generally accepted norm.

In bone material from *ramus mandibulae* anterior and posterior part trabecules contained chaotically localized collagenous fibres and unevenly located osteon structures. In osteon canals were seen irregular connective tissue ingrowth and blood vessel sclerotization. *Tuber maxillae* in bone tissue also had irregular bone mineralization, as well as there was seen osteon canal obliteration and connective tissue proliferation.

Control group patients' bone tissue in tissue material from *tuber maxillae, ramus mandibulae* anterior and posterior parts were without marked structural changes and with a histologic picture corresponding to the general norm.

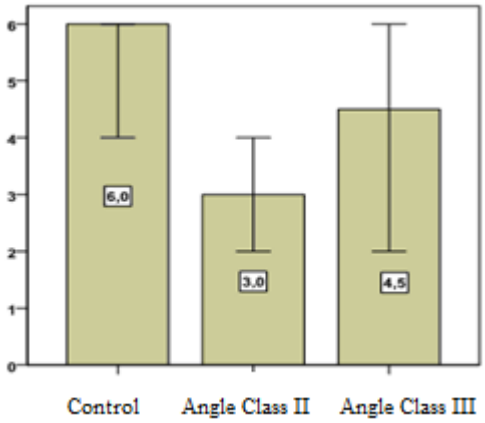
Immunohistochemical findings

Expression of growth factors and their receptors

Transformating growth factor β (TGF-β)

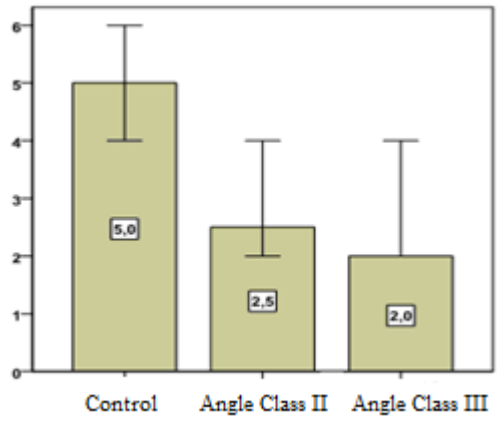
The most pronounced TGF-β expression from *tuber maxillae* was observed in control group patients (+++ or a lot of positive structures were seen in the visual field).

In class III was seen to have slightly lesser expression or averagely many to many positive structures in the visual field (++/+++), but in class II few to average positive structures in the visual field (+/++).After independent selection Kruskal-Wallis test in patients' groups TGF-β expression in bone tissue from *tuber maxillae* did not statistically significantly differ (χ^2 = 3,218; df = 2; p = 0,200). TGF-β expression median value and 95% significance interval in patients' groups is seen in Picture 1.



Picture 1. TGF-β exspression in *tuber maxillae* (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

Analyzing TGF-β expression from *ramus mandibulae* anterior part we saw that the most pronounced growth factor expression was in the control group or averagely many to many positive structures in the visual field (++/+++), but in class II and class III patients we found a slight amount of positive cells in the visual field (+ - +/++). After an independent selection Kruskal-Wallis test in the patient groups TGF-β expression in *ramus mandibulae* anterior part bone tissue did not statistically significantly differ (χ^2 = 4,156; df = 2; p = 0,125). TGF-β expression median value and 95% significance interval in patient groups is seen in Picture 2.

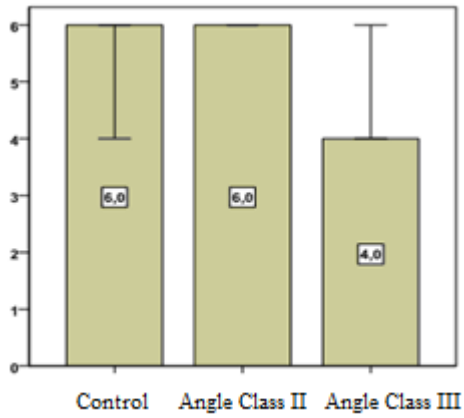


Picture 2. TGF-β expression in *ramus mandibulae* anterior part in connection with the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

In *ramus mandibulae* posterior part in all study groups we found a few positive structures in the visual field (+), but in some cases we did not observe any positive cells in the visual field (0). After an independent selection Kruskal-Wallis test TGF-β expression in *ramus mandibulae* posterior part between the patient groups did not statistically significantly differ (χ^2 = 0,340; df = 1; p = 0,560).

TGF-β expression in epithelium was greatly pronounced since practically in all tissue samples we found a lot of positive cells in the visual field. After an independent selection Kruskal-Wallis test TGF-β expression in epithelium in the patient groups did not statistically significantly differ (χ^2 = 4,749; df = 2; p = 0,093) and median in all groups was 6 (+++) – a lot of cells.

In tissue material from the lower jaw transitory fold in the second molar region the transforming growth factor β expression in connective tissues in the class III patient group had average to many positive structures in the visual field (++), but a lot of positive structures in the visual field were seen in class II and control groups (+++). After an independent selection Kruskal-Wallis test TGF-β expression in the connective tissues did not statisticaly significantly differ (χ^2 = 5,263; df = 2; p = 0,072). TGF-β expression medial value and 95% significance interval in the patient groups is seen in Picture 3.

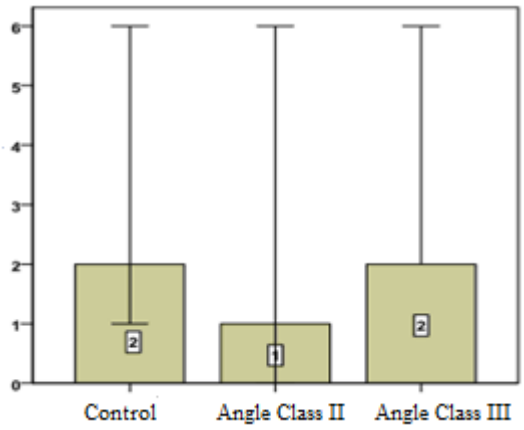


Picture 3. TGF-β expression in the connective tissues in connection with the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

Bone morphogenetic protein 2/4 (BMP2/4)

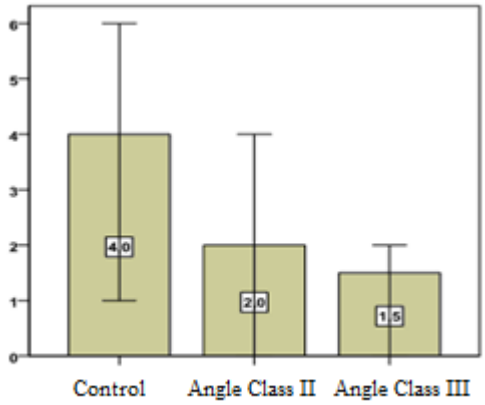
Both in the control group patients and class III patients BMP2/4 expression in the tissue material from *tuber maxillae* was similar (+ or few positive structures in the visual field). For

class II we observed a lesser expression or some positive structures in the visual field (0/+). After an independent selection Kruskal-Wallis test BMP2/4 expression in bone samples from *tuber maxillae* class II, class III and the control group patients did not statistically significantly differ (χ^2 = 2,095; df = 2; p = 0,351). BMP2/4 expression median value and 95% significance interval in the patient groups is seen in Picture 4.



Picture 4. BMP2/4 expression in *tuber maxillae* in relation to the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

The most pronounced BMP2/4 expression in *ramus mandibulae* anterior part was observed in the control group patients (++ or average to many positive structures in the visual field). In class II group we observed a lesser BMP2/4 identification intensity (+ or few positive structures in the visual field), but in class III we found a slightly lesser BMP2/4 expression (0/+ - + or from some positive structures to a slight amount of positive structures in the visual field). After an independent selection Kruskal-Wallis test BMP2/4 expression in *ramus mandibulae* anterior part in the patient groups did not statistically significantly differ (χ^2 = 3,640; df = 2; p = 0,162). BMP2/4 expression median value and 95% significance interval in the patient groups is seen in Picture 4.



Picture 5. BMP2/4 expression in *ramus mandibulae* anterior part (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

BMP2/4 expression in ramus *mandibulae* posterior part class II and class III groups was with a few positive structures in the visual field (+), but in some cases we observed also a lot of positive cells in the visual field (+++). In patient groups after an independent selection Kruskal-Wallis test BMP2/4 expression in *ramus mandibulae* posterior part did not statistically significantly differ (χ^2 = 0,556; df = 2; p = 0,456).

BMP2/4 expression in mucosal epithelium was not seen either in control, or class II patients, as well as not in majority of class III patients. Only 2 class III patients were seen to have a lot of positive structures in the visual field (+++).

A slight BMP2/4 expression was observed in the control group (0 - 0/+). Also in class II and class III groups we did not find any positive cell in the vision field (0), however, in some class II and class III patients were found to have a lot of positive cells in the visual field (+++), but in 3 class III patients we found also average to many positive structures in the visual field (++).

Fibroblast growth factor receptor 1 (FGFR1)

Analyzing FGFR1- containing relative amount of structures in the bone tissue from *tuber maxillae*, we found that class III patients were seen to have average to many positive structures in the visual field (++), but in class II group we found a slight amount (+) positive structures in the visual field. After an independent selection Kruskal-Wallis **test FGFR1 expression in *tuber maxillae* class II, class III and the control group patients differed statistically significantly** (χ^2 = 5,347; df = 1; p = 0,021).

Analyzing FGFR1 expression in bone tissue from *ramus mandibulae* anterior part we found equal this growth factor's receptor expression both in class II, and class III groups (+ or few positive structures in the visual field). After an independent selection Kruskal-Wallis test FGFR1 expression in *ramus mandibulae* anterior part in patient groups did not statistically significantly differ (χ^2 = 3,275; df = 1; p = 0,070).

FGFR1 expression in *ramus mandibulae* posterior part in class II patient group was with a few positive structures in the visual field (+), but in class III cases we observed few to average positive structures in the visual field (+/++). After an independent selection Kruskal-Wallis test FGFR1 expression in *ramus mandibulae* posterior part patient groups did not statistically significantly differ (χ^2 = 1,025; df = 1; p = 0,311).

The most pronounced fibroblast growth factor's receptor 1 (FGFR1) expression in the tissue material epithelium from the lower jaw transitory fold in the second molar region was observed in class III group patients, since in all analyzed samples we found a lot of positive structures in the visual field (+++). In class II group we observed a slightly lesser FGFR1 expression intensity (++ or average to many positive structures in the visual field). In two class II cases not a single (0) positive structure was seen in the visual field.

After an independent selection Kruskal-Wallis **test FGFR1 expression in epithelium in the patient groups differed statistically significantly** (χ^2 = 7,950; df = 1; p = 0,005).

Analyzing FGFR1 expression in the mucosal connective tissues in class II and class III groups, we found a pronounced FGFR1 expression, since practically in all analyzed samples we found a lot of positive structures in the visual field. After an independent selection Kruskal-Wallis test FGFR1 expression in connective tissues in the patient groups did not statistically significantly differ (χ^2 = 3,592; df = 1; p = 0,058).

Vascular endothelial growth factor (VEGF)

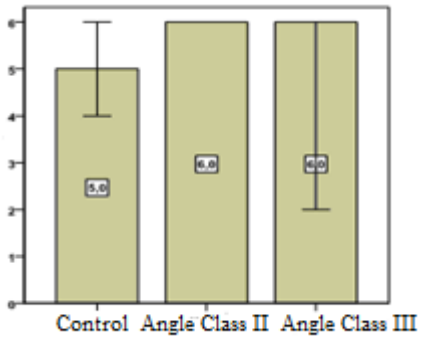
Vascular endothelial growth factor (VEGF) expression was in total detected in 38 samples, mainly in the epithelium and connective tissues, and only in one sample there were seen a few positive structures in the epithelium (+). In the rest of cases the expression of this factor was not observed.

Bone extracellular matrix protein expression

Osteocalcin (OC)

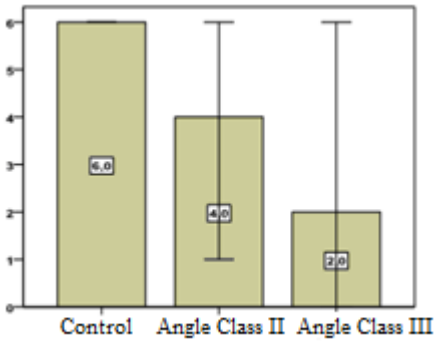
Analyzing the tissue material from *tuber maxillae*, we found a pronounced osteocalcin expression in class II and class III patients group (+++ or a lot of positive structures in the

visual field), but slightly lesser OC expression in the control patient group (++/+++ or averagely many to many positive structures in the visual field). After an independent selection Kruskal-Wallis test osteocalcin expression in the tissues from *tuber maxillae* in the patient groups did not statistically significantly differ (χ^2 = 1,797; df = 2; p = 0,407). Osteocalcin expression median value and 95% significance interval in the patient groups is seen in Picture 6.



Picture 6. Osteocalcin expression in *tuber maxillae* in relation to the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

The most pronounced OC expression in bone tissue from *ramus mandibulae* anterior part we found in the control group (+++). In class III group we observed average to many positive structures in the visual field (++), but in class II group – a slight amount of positive cells (+) in the visual field.



Picture 7. Osteocalcin expression in *ramus mandibulae* anterior part in relation to the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

After an independent selection Kruskal-Wallis test osteocalcin expression in *ramus mandibulae* anterior part in the patient groups did not statistically significantly differ (χ^2 = 5,517; df = 2; p = 0,063). Osteocalcin expression median value and 95% significance interval in the patient groups is seen in Picture 7.

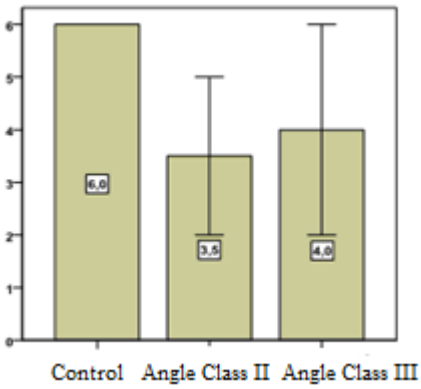
OC expression in *ramus mandibulae* posterior part was determined only in skeletal class II patient group. 5 measurements were done and it was found that in 2 patients the expression evaluation was 0 (not a single positive structure was seen in the visual field), in one case – + (a slight amount of positive structures in the visual field) and in two cases – +++ (a lot of positive structures in the visual field).

Osteopontin (OP)

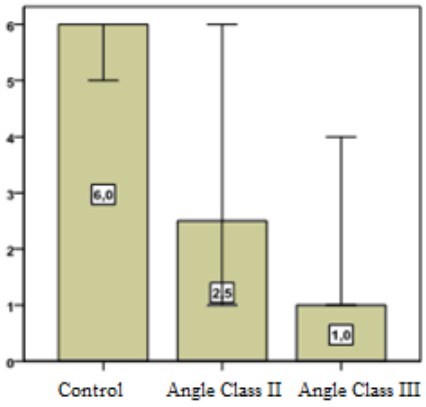
Analyzing OP expression in bone tissue from *tuber maxillae,* we found a lot of positive structures in the visual field in the control group patients (+++). Comparing with a control group, class II and class III patient groups OP expression was less pronounced – in class II group we found few to average positive structures in the visual field, but in class III group averagely many to many positive structures in the visual field. After an independent selection Kruskal-Wallis test **osteopontin expression in *tuber maxillae* patient group differed statistically significantly** (χ^2 = 5,980; df = 2; p = 0,050). Osteopontin expression median value and 95% significance interval in the patient groups is seen in Picture 8.

In the tissue material from *ramus mandibulae* anterior part the osteopontin expression in the control group patients was most pronounced (+++ or a lot of positive structures in the visual field), but a slight amount of positive structures in the visual field was seen in class II group (+ - +/++) and class III group (0/+). After an independent selection Kruskal-Wallis test the osteopontin expression in *ramus mandibulae* anterior part in the patient groups did not statistically significantly differ (χ^2 = 5,865; df = 2; p = 0,053). Osteopontin expression median value and 95% significance interval in the patient groups is seen in Picture 9.

Osteopontin expression in *ramus mandibulae* posterior part was determined in class II patient group. 8 measurements were done and it was found that in one patient the expression evaluation was 0 (none of positive structure were seen in the visual field), in three patients – + (few positive structures in the visual field), in one patient – +/++ (few to average positive structures in the visual field), in one patient – ++ (average to many positive structures in the visual field) and in two patients – +++ (a lot of positive structures in the visual field).



Picture 8. Osteopontin expression in *tuber maxillae* in relation to the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).



Picture 9. Osteopontin expression in *ramus mandibulae* anterior part in relation to the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

Matrix metaloproteinase 2 (MMP2)

Analyzing MMP2 expression in *tuber maxillae* we found that only two class III_group patients were seen to have a slight amount of positive structures in the vision field but_in all the rest of cases no positive structure was found in the visual field. After an independent selection Kruskal-Wallis test MMP2 expression in *tuber maxillae* patient groups did not statistically significantly differ (χ^2 = 0,618; df = 2; p = 0,734).

Similarly like in the tissue samples from *tuber maxillae,* we found also in bone tissue from *ramus mandibulae* anterior part weakly pronounced MMP2 expression, since in all class III patient samples there was not found a single positive structure in the visual field, but in class II group only in two cases were found some positive structures and in one sample averagely

many to many positive structures in the visual field. After an independent selection Kruskal-Wallis test **MMP2 expression in *ramus mandibulae* anterior part in the patient groups statistically significantly differed** (χ^2 = 7,930; df = 2; p = 0,019).

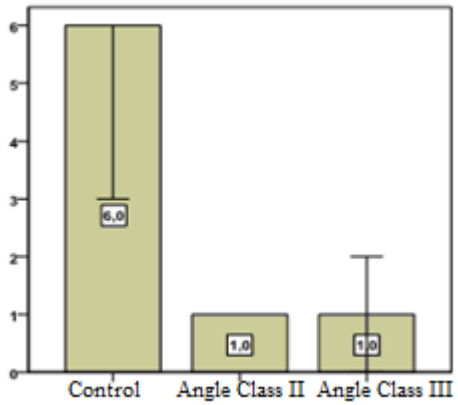
In neither of the analyzed samples was found MMP2 expression in the tissue material from *ramus mandibulae* posterior part.

Both in mucosal epithelium and in the connective tissues in all patient groups we found weakly pronounced MMP2 expression. After an independent selection Kruskal-Wallis test MMP2 expression in mucosal epithelium (χ^2 = 1,000; df = 2; p = 0,607) and in the connective tissues (χ^2 = 1,906; df = 2; p = 0,386) in the patient groups did not statistically significantly differ.

Barx1, msx2 and wnt1 gene proteins

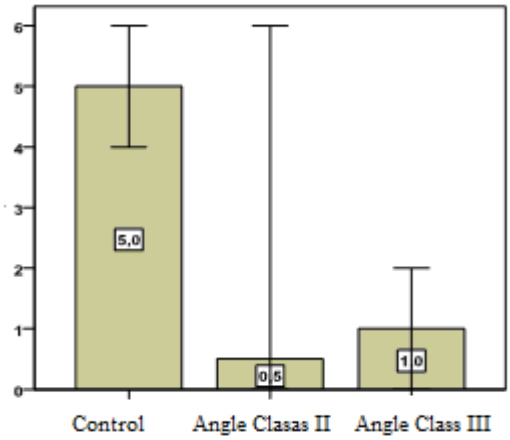
Analyzing barx1 gene protein immune reactivity in the tissue samples from *tuber maxillae* we could find that rich barx1 gene protein expression was seen in the control patient group (+++ or a lot of positive structures in the visual field), but in class II and class III patient groups these gene protein expression was much weaker (0/+ or rare occurance of positive structures in the visual field).

After an independent selection Kruskal-Wallis test **barx1 expression in *tuber maxillae* patient groups differed statistically significantly** (χ^2 = 7,406; df = 2; p = 0,025). Barx1 expression median value and 95% significance interval in the patient groups is seen in Picture 10.



Picture 10. Barx1 gene protein expression in *tuber maxillae* in relation to the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

The most pronounced expression of barx1 gene protein in the tissue samples from *ramus mandibulae* anterior part was in the control group (+++ or a lot of positive structures in the visual field). In class II and class III patient groups barx1 gene protein expression was less pronounced (0/+ or rare occurance of positive structures in the visual field). After an independent selection Kruskal-Wallis test **barx1 expression in *ramus mandibulae* anterior part in the patient groups differed statistically significantly**(χ^2 = 6,747; df = 2; p = 0,034). Barx1 exspression median value and 95% significance interval in the patient groups is seen in Picture 11.



Picture 11. Barx1 gene protein expression in *ramus mandibulae* anterior part in relation to the patient group (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field).

Analyzing barx1 gene protein expression in *ramus mandibulae* posterior part we found that both in class II and class III group patients in the majority of cases no single positive structure was found in the visual field and only in one class II patient's tissue sample we found a lot of positive structures in the visual field (+++). After an independent selection Kruskal-Wallis test barx1 expression in *ramus mandibulae* posterior part in the patient groups did not statistically significantly differ (χ^2 = 0,933; df = 1; p = 0,334). We found that barx1 gene protein expression in mucosal epithelium and in the connective tissues in the control group, as well as also in class II and class III patient group varied from 0 (no single positive structure in the visual field) to +++ (a lot of positive structures in the visual field), but + (few positive structures in the visual field) was found in majority of cases. After an independent selection Kruskal-Wallis test barx1 expression in the mucosal epithelium (χ^2 = 0,552; df = 2; p =

0,759) and in the connective tissues (χ^2 = 4,542; df = 2; p = 0,103) in the patient groups did not statistically significantly differ.

Analyzing msx2 gene protein expression in *tuber maxillae* we found that in majority of cases not a single positive structure was observed in the visual field (0) and only in four class III patient tissue samples we found some positive structures in the visual field (0/+). After an independent selection Kruskal-Wallis test msx2 expression in *tuber maxillae* patient groups did not statistically significantly differ (χ^2 = 1,107; df = 2; p = 0,575). In *ramus mandibulae* anterior part msx2 gene protein expression was found in 5 patients of class III group, but in the control and class II group – only one patient from the group. After an independent selection Kruskal-Wallis test msx2 expression in *ramus mandibulae* anterior part in the patient groups did not statistically significantly differ (χ^2 = 0,605; df = 2; p = 0,739). In neither of the analyzed samples, either class II, or class III, as well as in the control group msx2 gene protein expression in *ramus mandibulae* posterior part was not found. Analyzing msx2 gene protein expression in the mucosal epithelium, we did not find any positive structure in the visual field in the control group patients. Comparing with the control group, class II and class III patient groups msx2 gene protein expression was more pronounced, because msx2 gene protein expression varied from +++ (a lot of positive structures in the visual field) to 0 (not a single positive structure), but 4 class III patients were found + or few positive structures in the visual field. After an independent selection Kruskal-Wallis test msx2 expression in the epithelium in the patient groups did not statistically significantly differ (χ^2 = 4,475; df = 2; p = 0,107). Similarly as in the mucosal epithelium, also in the connective tissues, msx2 gene protein expression in the control group was not found (0), but in class II and class III patient groups msx2 gene protein expression was moderate + (few positive structures in the visual field). Msx2 expression in the connective tissues after an independent selection Kruskal-Wallis test in the patient groups did not statistically significantly differ (χ^2 = 5,371; df = 2; p = 0,068).

In neither of class II and class III patients' analyzed samples could be found wnt1 gene protein expression in the bone tissue from *tuber maxillae*, but in the control group the expression varied from 0/+ (rare occurance of positive structures in the visual field) to ++/+++ (averagely many to many positive structures in the visual field). After an independent selection Kruskal-Wallis test **wnt1 expression in *tuber maxillae* patient groups differed statistically significantly** (χ^2 = 19,860; df = 2; p = 0,001). In bone tissue from *ramus mandibulae* anterior part in neither of the analyzed samples was found wnt1 gene

protein expression. Class II, class III and the control group patients wnt1 gene protein expression in *ramus mandibulae* posterior part was found only in one case – some positive structures in the visual field, but in all the rest of cases – no positive structure was found in the visual field. Analyzing wnt1 gene protein expression, we found that both in the mucosal epithelium, and in the connective tissues in class III and the control group patients' tissue samples, on average there was a few positive structures in the visual field (+). In class II patients' samples only in two cases were found a few positive structures, but in all the rest of cases there was not found a single positive structure in the visual field. After an independent selection Kruskal-Wallis test **wnt1 expression in the epithelium in the patient groups differed statistically significantly** uχ^2 = 6,744; df = 2; p = 0,034), but wnt1 expression in the connective tissues in the patient groups did not statistically significantly differ (χ^2 = 2,344; df = 2; p = 0,310).

Summarizing the studied growth factors, bone extracellular matrix proteins, degeneration enzyme and gene proteins expression in the jaw bones and the surrounding soft tissues in skeletal class II, class III and the control patient groups (see Table 5), we can see that the expression of these biological markers is rather variable, however, in all the study groups the most pronounced is the expression of those markers, which according to the literature data available, are mainly responsible for the bone remodelling. We observed quite a rich expression of transforming growth factor β, bone morphogenetic protein 2/4, osteocalcin and osteopontin in the studied group's bone tissue from *tuber maxillae,* as well as from *ramus mandibulae* anterior and posterior parts. Analyzing FGFR1, we can see that the expression of this growth factor's receptor is more pronounced just in the soft tissues from the lower jaw gingival transitory fold in the second molar region in comparison to the expression in the bone tissue. Less pronounced is barx1, msx2 and wnt1 gene protein expression, while vacular endothelial growth factor and degeneration enzyme MMP2 was found only in some tissue samples, which were obtained from patients with class II and class III dentofacial deformations, but in the control group in neither tissue sample were found positive structures against VEGF and MMP2. We can also see that in the control group the expression of biological markers in *tuber maxillae* and *ramus mandibulae* anterior is rather similar, but in class III patient group a richer expression is observed just in the bone tissue from *tuber maxillae* and *ramus mandibulae* posterior parts, while in class II group's tissue samples, that which is obtained from *tuber maxillae* and *ramus mandibulae* anterior part.

Table 5. Mean indices of growth factors, bone extracellular matrix proteins, degeneration enzymes and gene protein expression in the jaw bones and soft tissues in the patient groups (0 – none of positive structures were seen in the visual field, 1 (0/+) - rare occurance of positive structures in the visual field, 2 (+) - few positive structures in the visual field, 3 (+/++) – few to average positive structures in the visual field, 4 (++) – average to many positive structures in the visual field, 5 (++/+++) – averagely many to many positive structures in the visual field, 6 (+++) – a lot of positive structures in the visual field, TM – *tuber maxillae*, RMAP – *ramus mandibulae* anterior part, RMPP – *ramus mandibulae* posterior part). By red colour are highlighted factors whose expression differed statistically significantly.

	Angle Class II					**Angle Class III**					**Control (Angle Class I)**				
	TM	RMAP	RMPP	Mucosa		TM	RMAP	RMPP	Mucosa		TM	RMAP	RMPP	Mucosa	
				Epithelium	Connective tissue				Epithelium	Connective tissue				Epithelium	Connective tissue
TGF-β	+/++	+/++	+	+++	+++	++/+++	+	+/++	+++	++	+++	++/+++	-	+++	+++
BMP2/4	0/+	+	+	0	+	+	+	+/++	+	+/++	+	++	-	0	0/+
FGFR1	+	+	+	++	+++	++	+	+/++	+++	+++	+	-	-	-	-
VEGF	0	0	0	0	0	0	0	0	0	0	0	0	-	0	0
OC	+++	++	+	-	-	+++	+	-	-	-	++/+++	+++	-	-	-
OP	+/++	+/++	+/++	-	-	++	0/+	-	-	-	+++	+++	-	-	-
MMP2	0	0/+	0	0	0	0/+	0	0	0	0/+	0	0	-	0	0
barx1	0/+	0/+	+	+/++	+	0/+	0/+	0/+	+	+	+++	++/+++	-	+	0/+
msx2	0	0/+	0	+	0/+	0/+	0/+	0	+	+	0	0/+	-	0	0
wnt1	0	0	0/+	+	0	0	0	0	0/+	+	+/++	0	-	++	+

Apoptosis findings

Analyzing the mean number of apoptopic cells in the bone tissue from *tuber maxillae* class II, class III and in the control patient groups, we found that a greater number of apoptotic cells were in the control group (mean value 25,89 ± 11,34 cells in the visual field). In class II and class III patients the number of apoptopic cells is practically equal and less than in the control group (mean values respectively are 9,42 ± 19,56 and 9,25 ± 9,53). Checking the statistic hypothesis on the equality of mean cell number in the patient groups by dispersion analysis, we acquired that the mean values in the groups were statistically significantly different ($F = 3,784$; $p = 0,038$).

Also in the tissue material from *ramus mandibulae* anterior part the apoptopic cell number in the control group is the greatest (mean value 28,44 ± 6,45), but in class II and class III patients the apoptopic cells are seen to be in a lesser amount. Similarly as in tissues from *tuber maxillae*, also in *ramus mandibulae* anterior part, comparing the mean apoptopic cell number in class II and class III patient groups, we found out that the results are similar (mean values respectively are 10,84 ± 10,52 and 11,86 ± 13,38). Checking the statistic hypothesis on the equality of mean cell number in the patient groups by dispersion analysis, we acquired that the mean values in the groups were not statistically significant ($F = 2,933$; $p = 0,067$). It is evident that only the mean value of the control group differs.

Number of apoptotic cells in *ramus mandibulae* posterior part was determined only in class II and class III patient groups (mean values respectively were 7,27 ± 11,74 and 5,73 ± 5,19 cells). Slightly lesser number of apoptotic cells is in class III patient group. According to an independent selection t test, the mean cell number in the groups did not statistically significantly differ ($t = 0,277$; $df = 14$; $p = 0,786$). Since the standard deviation for selections is comparable to the mean value, which is a sign that the data distribution does not correspond to a normal probability distribution, consequently the non-parametric analysis was done as well. For the comparison of the mean range of two independent selections, we used Mann-Whitney test and obtained that in class II and class III patient groups the mean ranges were 7,68 and 10,30 respectively, and they did not statistically significantly differ ($Z = 1,026$; $p = 0,305$).

Analyzing the mean number of apoptotic cells, we found out that both in the mucosal epithelium and in the connective tissues in class III and class II group patients' tissue samples the number of apoptosis-affected cells was approximately equal, though slightly lesser than in the control group.

6.3.6. Cross-correlations of immunohistochemical results

The acquired results of immunohistochemical studies were analyzed to state the cross-correlations and there were found several correlations of the relative amount of morphologic parameters.

In the bone tissue material from *tuber maxillae* there correlated several parameters (see Table 6). Statistically significant close positive correlation was found between wnt1 gene protein and barx1 gene protein expression ($p = 0{,}001$; $r = 0{,}665$). In the same way we found a statistically significant close positive correlation between wnt1 gene protein expression and apoptosis ($p = 0{,}001$; $r = 0{,}845$). We found that by increase of wnt1 gene protein expression in tissues from *tuber maxillae,* there increases also barx1 gene protein expression and there is evidence of a greater number of apoptotic cells.

Table 6. The cross-correlations between growth factors, bone extracellular matrix proteins, degeneration enzymes and gene protein expression in *tuber maxillae.* (r - correlation coefficient; p - statistical significance, * - $p \leq 0{,}05$; ** - $p \leq 0{,}001$).

		TGF-β TM	BMP2/4 TM	FGFR1 TM	OC TM	MMP2 TM	barx1 TM	msx2 TM	wnt1 TM	TUNEL TM
BMP2/4 TM	r	**0,415**	1							
	p	***0,035**								
FGFR1 TM	r	-0,070	0,077	1						
	p	0,764	0,748							
OC TM	r	**0,512**	0,288	-0,449	1					
	p	***0,036**	0,279	0,166						
MMP2 TM	r	0,087	0,308	-0,309	0,270	1				
	p	0,701	0,175	0,213	0,397					
barx1 TM	r	0,227	0,187	0,033	0,379	0,255	1			
	p	0,323	0,429	0,897	0,251	0,264				
msx2 TM	r	-0,108	-0,158	0,321	0,083	-0,157	-0,286	1		
	p	0,642	0,506	0,194	0,808	0,496	0,208			
wnt1 TM	r	0,239	0,306		0,203	-0,114	**0,665**	-0,171	1	
	p	0,297	0,190		0,550	0,622	****0,001**	0,459		
TUNEL TM	r	0,287	0,249	**0,615**	0,272	-0,296	**0,736**	-0,082	**0,845**	1
	p	0,249	0,352	***0,025**	0,393	0,325	****0,004**	0,790	****0,001**	

Analyzing the cross-correlations we found out that there existed a moderately close statistically significant correlation between the transforming growth factor β (TGF-β) and the bone morphogenetic protein 2/4 (BMP2/4) expression ($p = 0{,}035$; $r = 0{,}415$), as well as between

TGF-β and osteocalcin expression (p = 0,036; r = 0,512). With the increase of the number of apoptotoc cells in *tuber maxillae* bone tissue, there in parallel increases also the fibroblast growth factor's receptor 1 (FGFR1) amount in the tissues, which gives the evidence to a moderately close statistically significant positive correlation (p = 0,025; r = 0,615).

Analyzing the correlations in the tissue material from *ramus mandibulae* anterior part (see Table 7), we found out statistically significantly close positive correlation between TGF-β and BMP2/4 (p = 0,008; r = 0,427), as well as TGF-β and barx1 (p = 0,001; r = 0,663). With the increase of TGF-β expression, there increases the number of apoptotic cells, since both of these indices correlated between each other with a statistically significant close correlation (p = 0,003; r = 0,548).

Table 7. The cross-correlations between growth factors, bone extracellular matrix proteins, degeneration enzymes and gene protein expression in *ramus mandibulae* anterior part (r - correlation coefficient; p - statistical significance, * - $p \leq 0,05$; ** - $p \leq 0,001$).

		TGF-β RMAP	BMP2/4 RMAP	FGFR1 RMAP	OC RMAP	MMP2 RMAP	barx1 RMAP	msx2 RMAP	wnt1 RMAP	TUNEL RMAP
BMP2/4 RMAP	r	**0,427**	1							
	p	****0,008**								
FGFR1 RMAP	r	-0,037	-0,073	1						
	p	0,836	0,691							
OC RMAP	r	**0,522**	0,246	-0,057	1					
	p	***0,018**	0,309	0,835						
MMP2 RMAP	r	0,083	**0,486**	-0,064		1				
	p	0,708	***0,019**	0,794	,000					
barx1 RMAP	r	**0,663**	0,280	-0,175	**0,874**	-0,103	1			
	p	****0,001**	0,207	0,488	***0,023**	0,658				
msx2 RMAP	r	-0,093	-0,161	-0,141	0,000	-0,029	-0,157	1		
	p	0,680	0,475	0,577	1,000	0,902	0,497			
wnt1 RMAP	r	**0,466**	**0,444**		0,603	-0,106	**0,705**	-0,067	1	
	p	***0,025**	***0,034**	0,000	0,205	0,638	****0,001**	0,768		
TUNEL RMAP	r	**0,548**	**0,502**	0,036	0,130	-0,251	**0,897**	-0,337	**0,719**	1
	p	****0,003**	****0,009**	0,865	0,595	0,387	****0,001**	0,239	****0,003**	

We also found that in the tissues from *ramus mandibulae* anterior part there exists a statistically significantly close positive correlation between the number of apoptotic cells and BMP2/4 expression (p = 0,009; r = 0,502). Statistically close positive correlation was found between gene proteins barx1 and wnt1 (p = 0,001; r = 0,705), as well as by the growth of the

number of apoptotic cells there increases barx1 (p = 0,001; r = 0,897) and wnt1 expression (p = 0,003; r = 0,719). Between some indices in *ramus mandibulae* anterior partl bone tissue we found a statistically significant moderately close positive correlation, for instance, between TGF-β and osteocalcin expression (p = 0,018; r = 0,522), as well as between the growth factor TGF-β and wnt1gene protein (p = 0,025; r = 0,466). In the same way there correlates wnt1 gene protein with BMP2/4 positive structure amount (p = 0,034; r = 0,444) and BMP2/4 expression with MMP2 expression (p = 0,019; r = 0,486).

The amount of the growth factor TGF-β in the studied patients' bone tissue from *ramus mandibulae* posterior part statistically significantly moderately closely correlates positively with FGFR1 expression (p = 0,041; r = 0,595). In class II, class III and the control group patients, who were observed a greater barx1 gene protein expression, there was also a greater wnt1 expression (p = 0,046; r = 0,746), comparing with the patients whose barx1 expression in *ramus mandibulae* posterior part was positive rarely or very little. Moderately close statistically significant correlation was found also between barx1 and the number of apoptotic cells (p = 0,028; r = 0,918).

In neither of the studied morphologic parameters we found any statistically significant, or moderately close positive correlations in the mucosal epithelium, acquired from the second molar region.

Also in the mucosal connective tissues we found only moderately close statistically significant positive correlation between the number of apoptotic cells and and wnt1 gene protein expression (p = 0,025; r = 0,542), as well as a moderately close statistically significant negative correlation between MMP2 expression and wnt1 gene protein expression (p = 0,014; r = -0,570), where with the decrease of MMP expression, there increases wnt1 expression and vice versa.

SUMMARY

Summaryzing the results of our study, we have to conclude that in morphogenesis the important growth factors and their receptors (TGF-β, BMP2/4, FGFR1 and VEGF), bone extracellular matrix proteins (OC, OP), degeneration enzymes (MMP2), gene proteins (barx1, msx2, wnt1) and apoptosis parameters are different in jaw bone growth zones in various dentofacial deformation groups, as well as changed skeletal class II and class III patients' bone tissue and surrounding soft tissue morphologic structure.Mucosal changes found in skeletal class II and class III patients, which are characterized by epitheliocyte foci-type vacuolization, cell

hyperplasia, basal membrane changes and inflammatory cell infiltration, probably point to difficulties of oral hygiene. In the bone tissue of patients under study there was noticed irregular mineralization, vascular sclerosis and connective tissue proliferation in osteon canals, not providing the jaw bones with a sufficient blood supply, thus, affecting the upper and lower jaw growth in patients with dentofacial deformations**.**

Our study results give proof that patients with skeletal dentofacial deformations have a greater amount of relative positive structures for important growth factors well-recognized in bone tissue morphogenesis, such as TGF-β and BMP2/4, and bone extracellular matrix proteins (OP and OC) with slight differences between classes, pointing to the importance of these morphologic markers in supportive tissue remodelling and the development of bone tissue in patients with dentofacial deformations.

FGFR1 similar and marked expression in oral mucosa in all group tissue samples proves the importance of this growth factor' receptor just in the soft tissues, therefore we can conclude that all in all, oral mucosa is characteristic of sufficient regenerative potential which can be estimated as being positive, because skeletal class II and class III patients are performed orthognatic operation with intraoral incisions and this factor's dominant expression will provide fast reconvalescence of the oral mucosa after the operation.

Not pronounced MMP2 expression and practically lack of VEGF expression in patients' tissues indicate that both have an insignificant role in skeletal class II and class III patients bone remodelling, while summarizing the data on gene protein relative positive structure amount in bone tissue and soft tissues, we can conclude that the most pronounced barx1 gene protein expression is prevalent in the mucosa of class II and class III patients, indicating to their compensatory and stimulating role in promotion of bone tissue development. Although the meaning of Msx family genes has been widely described in the development of different congenital maxillofacial pathologies, however, in our study these genes' protein expression was comparatively insignificant and similar to wnt1 gene protein expression, which means that these markers in skeletal class II and class III dentofacial deformation morphopathogenesis have insignificant importance.

The greatest number of apoptopic cells in the control patients' bone tissue and soft tissues, but smaller and relatively equal in apoptosis marked cell number in skeletal class II and class III patients indicate to a destroyed balance between cell proliferation, differentiation and apoptosis in patients with dentofacial class II and class II deformations.

8. CONCLUSIONS

1. In accordance with the cephalometric data, the jaw bone morphologic picture of skeletal class II and class III patient dentofacial deformation is variable.

2. In skeletal class II and class III patients, who are undergoing orthognatic treatment their gingival epithelium for the orthognatic operation time is characterized by epitheliocyte vacuolization, cell hyperplasia, basal membrane changes and inflammatory cell infiltration at subepithelial verrucous layer.

3. Class II and class III patients' bone tissue is characterized by irregular mineralization, vascular sclerosis and connective tissue proliferation in the osteon canals.

4. In patients with skeletal dentofacial deformations the greater relative positive structure amount in bone tissue morphogenesis is played by significant growth factors, such as TGF-β and BMP2/4 with slight differences between class II and class III.

5. From bone tissue in *tuber maxillae* region the greater TGF-β and BMP2/4 expression is seen in class III and control groups, comparing to class II, which, probably, suggests to a potentially probable, yet not expressed bone growth in these region.

6. In r*amus mandibulae* anterior part the expression of significant factors in bone tissue growth (TGF-β un BMP2/4) is higher in the control group and class II patients, while in *ramus mandibulae* posterior part higher expression of TGF-β and BMP2/4 is in class III patients, comparing to class II, which indicates to a preserved growth potential in these jaw bone regions.

7. FGFR1 similar expression and practically lack of VEGF expression in patients' bone issue confirm both as insignificant factors in skeletal class II and class III patients bone remodelling. The same can be referred also to MMP2 unpronounced expression in the class II, class III and control groups.

8. More active bone extracellular matrix protein (osteocalcin and osteopontin) expression in *tuber maxillae* region both in class II and class III patient groups and different expression in *ramus mandibulae* anterior part, prove to the bone mineralization and metabolism activity changes, which, perhaps, characterize just these dentofacial deformations.

9. Barx1 expression prevalently in the mucosa in class II and class III patients, which differs from the control group's dominating expression in bone tissue, indicates, perhaps to the stimulating effect of soft tissue expressed factors on the bone tissue.

10. Amount of reduced and similar number of apoptotic cells in class II and class III patients suggest to the disorders of apoptotic process in orthognatic surgery patients.

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ABBREVIATIONS USED IN THE WORK

Abbreviations	Interpretation
BMP2/4	Bone morphogenetic protein 2/4
FGFR1	Fibroblast growth factor receptor 1
MMP2	Matrix metalloproteinase 2
OC	Osteocalcin
OP	Osteopontin
p	Statistical significance
RMAP	*Ramus mandibulae* anterior part
RMPP	*Ramus mandibulae* posterior part
TGF-β	Transforming growth factor β
TM	*Tuber maxillae*
TUNEL	Terminal dezoxynucleotidyl transferase mediated dUTP nick – end labeling
VEGF	Vascular endothelial growth factor