Rīgas Stradiņa universitāte

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QUANTITATIVE ASSESSMENT OF SIGNALLING MOLECULES IN HUMANS INTERRADICULAR SEPTUM TISSUES OF DIFFERENT AGE GROUPS

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

Speciality – Orthodontics

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Secretary of Doctoral Council:
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CONTENTS

ABBREVIATIONS USED IN THE PAPER

Abbreviation	Term in English	Explanation in Latvian
ANOVA	Analysis of Variance	Dispersiju analīze
BMP	Bone morphogenetic protein	Kaula morfoģenētiskais proteīns
BMP2/4	Bone morphogenetic protein 2/4	Kaula morfoģenētiskais proteīns 2/4
CSF	Colony stimulating factor	Koloniju stimulējošais faktors
ECM	Extracellular matrix	Ekstracelulārā matrice
IL	Interleukin	Interleikīns
FACIT	Fibril Associated Collagens with Interrupted Triple helices	Fibrilu saistītais kolagēns ar pārtrauktām trīskāršām spirālēm
bFGF	base Fibroblast growth factor	bāziskais Fibroblastu augšanas faktors
FGFR1	Fibroblast growth factor receptor 1	Fibroblastu augšanas faktora receptors 1
GF	Growth factor	Augšanas faktors
LDL	Low density lipoproteins	Zema blīvuma lipoproteīni
IGF	Insuline-like growth factor	Insulīnam līdzīgais augšanas faktors
MMP	Matrix metalloproteinase	Matrices metalproteināze
NF-κB	Nuclear factor-kappa B	Nukleārais faktors kapa B
NGF	Nerve growth factor	Nervu augšanas faktors
OC	Osteocalcin	Osteokalcīns
OPG	Osteoprotegerin	Osteoprotegerīns
p	Statistical significance	Būtiskuma (nozīmības) līmenis – varbūtība, kas ir spēkā statistiskajā testā izvirzītā nulles hipotēze
PDS	Periodontal ligaments	Periodonta saites
RANK	Receptor activator of NF-kappaB	NF-kappaB receptora aktivators
RANKL	Receptor activator of NF-kappaB ligand	NF-kappaB liganda receptora aktivators
TGFβ	Transforming growth factor β	Transformējošais augšanas faktors β

TIMP	Tissue inhibitor of matrix metalloproteinase	Matrices metaloproteināzes inhibītors
TNF-α	Tumour necrosis factor α	Tumora nekrozes faktors α
TUNEL	Terminal dezoxynucleotidyl transferase – mediated dUTP nick – end labeling	Tdt – gala dezoksinukleotīdtransferāze un digoksigēna–marķēti nukleotīdi
VEGF	Vascular endothelial growth factor	Vaskulārais endoteliālais augšanas faktors

1. TOPICALITY OF THE STUDY

When teeth move or physiologically migrate, the reaction of the surrounding and involved tissues is comparatively alike. However, when teeth are moved orthodontically, the speed of this reaction is definitely higher, reconstruction of the surrounding tissues and bone remodelling occurs significantly faster as the force applied orthodontically is large and almost uninterrupted. The knowledge about the processes taking place in tissues and their structures has been not fully researched yet (Graber, 2005). With the emergence of new methods it is possible to study the occurring processes more in-depth and to examine their action mechanisms on the molecular level. Dentofacial anomalies cause the patient functional, aesthetic and psychological problems. Bone as an organ is in a continuous remodelling process owing the mutual interaction of osteoblasts and osteoclasts. With the participation of other cells, the continuity of this process is ensured. Therefore, apoptosis has a great importance, since in order to have a place for something new, this place should be vacated by the old. Apoptosis as physiologically programmed cell death is a universal mechanism, established by nature that works smoothly under normal circumstances.

Orthodontic treatment is a combined and often also an interdisciplinary process, this can be particularly observed when treating already mature patients. It consists of orthodontic treatment with fixed braces system and, at times, periodontological, prosthetical and surgical treatment. Orthodontic treatment is usually started at the age of 12-14, when a permanent occlusion is established. Both boys and girls at this age may have a different occlusion development phase, which is linked to the reach of pubertal age, it ranges from 10.5 to 11.5 years and 11.5 to 12.5 years, respectively (Proffit, 2007).

There is no age limit for orthodontic treatment unless there are conditions that may influence the outcome of the treatment, however, with age regularity exists regarding the duration of the treatment which may have a connection with the factors involved in the remodelling process, their amount and activity.

Determination of growth factors, bone extracellular matrix proteins, degeneration enzymes and apoptosis in the bone tissues and soft tissues adjacent to them would give additional information on substances involved in the remodelling process and on morphogenesis of patients' individual bone structures.

Adapted biochemical response to the applied orthodontic force is a very complicated process. Several closely related reactions taking place in PDS and alveolar bone cells transform the mechanical strength into molecular signals and ensure orthodontic teeth movement. Osteoclasts and osteoblasts are able to restore homeostasis caused by the force applied to the teeth.

Bone adaptation to orthodontic forces is dependent on normally functioning genes in osteoclasts and osteoblasts that provide the necessary synthesis of proteins in the right place and time.

Current evidence suggests that mechanotransduction starts from the focal adhesions, which link the extracellular matrix with the cytoskeleton. Mechanically induced remodelling is implemented through a series of feed-back mechanisms involving the synthesis of cytokines, such as IL-1, IL-6 and receptor activator of nuclear factor κ B ligand (RANKL) synthesized by the osteoblast and fibroblast lineages. The synthesis is regulated by autocrines and paracrines, an expression of transcription factors, i.e., cytokines, growth factors, enzymes and the molecules involved in the differentiation, proliferation, and function of mesenchymal and other cell types (Masella, 2006).

Orthodontic tooth movement depends on the size of the applied force, direction and duration (Tanne, 1989). From the point of view of mechanics, the first reaction to orthodontic forces is PDS lesion, and lesion of the surrounding alveolar bone due to the pressure and the applied force to the traction place. This causes intra-alveolar tooth movement and bending of the surrounding alveolar bone (Cattaneo, 2005).

Different biological response of patients' tissues to mechanical irritation is directly dependent on the population, genome and synthesized proteins of their PDS and

alveolar bone cells. Only by achieving precise effect on target genes, cells and tissues it is possible to promote the introduction of safe and effective genetic engineering and the tissue growth led by stem cells (Malcolm, 2006).

During orthodontic treatment the initial changes in periodontal tissues on the side of pressure are divided into the initial and secondary phase, namely, hyalinisation and bone resorbtion. The degree of hyalinisation is dependent on the applied force. Therefore, great force causes bone necrosis and a wide area of hyalinisation that is separated from the necrotic area as it contains newly formed fibres and blood capillaries. Molecular mechanisms that separate the hyalinised tissues from the necrotic tissues are not fully clear, but it is likely that they determine the changes of cell apoptosis and necrosis. Some studies prove that apoptotic osteocytes adjacent to hyalinised periodontal ligaments show necrosis-like properties during initial tooth movement (Hayama et al., 2002). Therefore, cell death is a significant biological process that takes place as a result of orthodontic tooth movement of periodontal tissue remodelling.

Artificially grown human PDS cells are widely used to study the effect of traction on gene expression both in mRNA and protein levels, as well as to explore separately genes of secondary and late effects. The results depend on the condition of cells, force type (interrupted, cyclic versus continuing), duration and cell culture. For instance, it has been reported that the cyclical load both stimulates (Saito et al., 1991; Shimizu et al., 1994) and inhibits (Long et al., 2001) IL-1β activity in PDS cells. While the prolonged traction forces applied to the PDS cells, stimulate the synthesis of MMP-1 (collagenase-1), MMP-2 (gelatinase-A) TIMP-1 and TIMP-2 (Bocato-Bellemin et al. 2000), centrifugation of PDS cell cultures increases mRNA level in MMP-1, but has no effect on type I collagen and TIMP synthesis (Redlich et al., 2004). Of all the currently available evidence it is possible to construct a hypothetical model that shows the PDS cell response to mechanical force on the traction side in case of orthodontic tooth movement (Meikle, 2006).

Currently some researchers use *in vitro* methods to study the osteablast and PDS cell response to pressure. Kanasaki et al. (2002) found that cell signals from compressed PDS fibroblasts stimulate osteoclastogenesis in periferal mononuclear blood cell

cultures and that RANKL mRNS expression increases there. However, the OPG synthesis remained unchanged. This implies that PDS is more significant in osteoclast differentiation and ortodontic tooth movement. In other studies, He et al. (2004) found that PDS cells, exposed to traction and pressure forces, are capable of receiving two different types of mechanical stimulation and they respond differently, i.e., with different extracellular matrix synthesis (type I collagen, fibronectin) and degradation (MMP-2, TIMP -2). Connective tissue degradation and bone resorption comprise the interaction of different cell types. To study these processes the most suitable are in vivo methods (see Figure 2), since the molecular level events cause cascade-type reactions, such as osteoclast and giant cell proliferation and activation (Mumm, 2004).

In rat tooth movement studies with *in situ* hybridization method, the increase of IL-1β and IL-6 mRNA (but not TNF-α) expression both from PDS cells and osteoblasts on the pressure side (Alhashimi et al., 2001) was found, as well as the synthesis of MMP-8 (collagenase-2) and MMP-13 (collagenase-3) (Takahashi et al. 2003).
RANKL, OPG and mRNA are widely synthesized in periodontal tissue osteoblasts and PDS cells (Ogasawara et al., 2004). The subsequent tooth movement, with the use of Waldo method (Waldo and Rothblatt, 1954), positive RANKL and RANK signals were found in multinuclear osteoclasts that ensure active bone resorption. In addition, IL-1β and TNF-α expression was observed in osteoclasts.

The necessity to identify treatment options that can stimulate the regeneration of periodontal tissues led to a series of studies on periodontal tissue response to growth factor stimuli (Graves, 1994).

The transforming growth factor beta (TGFβ) stabilizes the collagen matrix, reducing the synthesis and secretion of fibroblast MMP. Macrophages and platelets are secreted by TGFβ. Fibroblasts in periodontal ligaments respond to parathyroid hormone similarly to osteoblasts, increasing the secretion of cyclic adenosine monophosphate. Fibroblast growth factors increase PDS proliferation and reduce the formation of alkaline phosphatase and, consequently, their ability to form mineralized tissues. PDS connective tissues have a high regenerative capacity. For regeneration of functional bonds, bone and cement formation similar to that of Sharpey's fibre fixation

is required. This particular tissue reorganization prevents PDS and bone fusion, which is the cause of ankylosis (Garant, 2003).

PDS remodelling process is similar to the healing process in connective tissues. New fibroblasts are created from perivascular progenitor cells adjacent to unchanged PDS fibroblasts. Fibroblast migration occurs in the fibrin and fibronectin network. New collagen fibres are formed chaotically, often without functional orientation and neither with fixation to adjacent tissues. Complete fibre reorganization occurs within a few weeks due to cell activity (Caton, 2000).

2. AIM OF THE STUDY

To identify the expression of degeneration, growth, mineralization and inflammation signalling molecules in the interradicular septum tissues, in order to substantiate the tissue remodelling possibilities during the orthodontic treatment process depending on age.

3. TASKS OF THE STUDY

1. Using histological staining with hematoxylin-eosin, to determine histological differences of bone tissues and periodontal tissues in the 1st (patients aged 12−14 years), 2nd (patients aged 15−22 years) and 3rd (patients aged 23−49 years) patient age groups.

2. Using immunohistochemistry method, to determine the signalling molecule expression in the alveolar bone and periodontal tissues in the 1st, 2nd and 3rd patient age groups:

a) Expression of growth factors and their receptors (transforming growth factor β (TGFβ), nuclear factor kappa B (RANKL), osteoprotegerin (OPG) fibroblast growth factor receptor 1 (FGFR1) and base fibroblast growth factor (bFGF) in bone and periodontal tissue samples.

b) To determine the expression of bone extracellular matrix proteins (osteocalcin (OC)) and extracellular matrix degradation enzymes matrix metalloproteinase 8 (MMP8), matrix metalloproteinase 9 (MMP9).

c) To determine the inflammatory factors of interleukin 6 (IL 6) expression.

3. Using the TUNEL method, to determine the frequency of apoptosis in alveolar bone and periodontal tissues in the 1st, 2nd and 3rd patient age groups.

4. To identify correlations between the expressions of growth factor, bone extracellular matrix protein, degeneration enzymes and apoptosis and patients' age.

4. NOVELTY OF THE STUDY

This is one of the unique studies in Latvia where human tissue samples are used to determine the differences of the expression of growth factors, bone extracellular matrix proteins, degeneration enzymes, inflammatory proteins and apoptosis, i.e., bone growth and remodelling indicator differences in the alveolar bone and periodontal tissues in relation to the patient's age determined.

5. MATERIALS AND METHODS

5.1. Study Group

The study group included 45 patients who had been diagnosed dentoalveolar anomalies and who needed orthodontic treatment. Patients were divided into 3 groups (see Table 5.1.1):

The 1st group of patients aged 12−14 years, a period characterized by puberty, when remodelling processes occur rapidly and metabolic activity reaches its highest point.

The 2nd group of patients aged 15−22 years marks the period during which the tissue maturation takes place, remodelling potential is high, but the metabolic activity starts declining gradually compared to the period of puberty.

The 3rd group of patients aged 23−49 years old is characterized by a mature tissue condition and stable metabolic activity that starts decreasing with years having consequent results.

Absolute and percentual distribution of patients in study classes according to gender is seen in table 5.1.1.

Table 5.1.1.

Absolute and percentual distribution of patients in study classes according to gender

Group	Gender				Total	
	Male		Female		Number	Per cent
	Number	Per cent	Number	Per cent		
1.	5	33.3	10	66.6	15	33.3
2.	7	45.0	8	55.0	15	33.3

(End of Table)

3.	9	65.4	6	34.6	15	33.3
Total	21		24		45	100.0

The following patients were not included in the study:

acute periodontitis;

severe overall illness history;

cleft lip and/or palate;

dentofacial syndromes;

skeletal asymmetries.

The tissues under the study were obtained within the project in the Centre of Maxillofacial Surgery of the Rīga Stradiņš University Institute of Stomatology from December 2010 to August 2013 by outpatient teeth extraction of orthodontic patients and planned orthognathic patients from the extracted maxillary premolar, molar and mandibular molar sites (according FDI sysytem 14, 16, 17, 18, 24, 26, 27, 28, 36, 37, 38, 46, 47, 48 on interradicular septum parts). The obtained tissue material was studied immunohistochemically to determine the presence of growth and apoptosis factors. The study used anonymos patient numbering, gender, age, medical history data, as well as clinical data (the extraction place and the extracted tooth diagnosis).

The applied tooth extraction technique was in accordance with the technology approved by the non-profit state JSC Health Statistics and Medical Technology Agency (Regulation of the Cabinet of Ministers No. 82, dated 01.02.2005.). Tissue pieces (1-2 sq mm thick) were taken from the tooth extraction places (interradicular septum) from the patients who, in relation to orthodontic treatment, had been prescribed tooth extraction without additional operative intervention.

5.2. Morphological Study Methods

Tissue fixation was carried out immediately in the surgery room. For this purpose a saturated picric acid solution (2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2)) was used which was in an Eppendorf bottle prepared in advance. This methodology was intended for immunohistochemical examinations, when particularly gentle structures, for instance, receptors, neuropeptides and factors with growth properties should have been fixed. It has been repeatedly tested in practice, and it takes only about 2 minutes for the operations support staff, therefore not disturbing the ongoing operation.

Afterwards the tissue pieces were taken to the Morphology laboratory of RSU AAI. Transportation of the tissues fixed in such a manner is not problematic and it does not require any specific preparation. Subsequently the tissues were treated according the chosen methodology.

The samples were examined under Leica DC300F microscope and further they were analyzed in the image analyzer system with the software Imagepro Plus 7.0 (systems and microscopes were acquired in 2003 and include a wide range of tissue analysis systems) located at the RSU Institute of Anatomy and Anthropology. The tissue blocks are stored in the archives of the RSU AAI Morphology Laboratory in a cabinet foreseen for the purpose.

In total 540 histological sections were prepared from the bone tissue samples of 45 people that were used for staining for morphological study with hematoxylin-eosin (H & E) (Aughey, Frye, 2001), TUNEL reaction (Negoescu et al., 1998), biotin – streptavidin immunohistochemical method (Hsu et al., 1981).

5.2.1. Sample Fixation and Preparation

The tissue material obtained during surgeries was fixed for 4 to 8 hours in a mixture containing 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2). After that tissue samples were washed in thyroid buffer (pH 7,6), containing

10% sucrose for 12 hours, and then tissues were embedded in paraffin and cut with microtome into 3 to 5 μm thick sections (Stefanini et al, 1967).

5.2.2. Sample Staining with Hematoxylin-Eosin

After deparaffinzation of the samples, they were stained for 7 minutes with hematoxylin. Afterwards the samples were rinsed with water for 10 minutes. Then followed 2-minute long staining with eosin, after which the samples were rinsed with water for a short while. Staining was followed by dewatering in an increasing concentration of alcohol and xylene solutions. The stained samples were coated with polystyrene and covered with a glass coverslip. As a result of staining, the basophilic parts of the cell were stained in blue violet tones, but acidophilus parts – from pink to dark red tones.

Leica DM or Leica DC300F microscope with 200x or 400x magnification was used for the light microscopic analysis.

5.2.3. Biotin-Streptavidin Immunohistochemical Method

The cut 3-5 μm sections were rinsed in phosphate buffer solution for 10 min. After that the sections were incubated with natural goat, mouse and rabbit 10% serum for 20 minutes to reduce background staining, and incubation with the primary antimatter was continued in a humid chamber at room temperature for 60 minutes (Hsu et al., 1981).

Using biotin and streptavidin immunohistochemical method matrix metalloproteinases MMP8 – 6 - 19Z: SC - 80206, 1:50; MMP9 – H - 129:SC-10737, 1:250 from Santa Cruz Biotechnology, Inc., California, USA), fibroblast growth factor receptor (FGFR, code ab10646, 1:100, Abcam, UK), transforming growth factor beta (TGFβ, code T1654, 1:1000, SigmaBioScience, USA), interleukin-6 IL6 code SC - 130326, 1:50 from Santa Cruz Biotechnology, Inc., California, USA), osteoprotegerin (OPG N - 20 SC - 8468; 1:40, Santa Cruz Biotechnology, Inc., California, USA), osteocalcin (OC code ab13418; Abcam; 1:100, Cambridge, UK), nuclear kappa B factor

protein (NKpB 105 code p105/p50[ab7971], Abcam; 1:100, Cambridge, UK), fibroblast growth factor receptor 1 (FGFR1, code 10646, working dilution 1:100, Abcam, UK) base fibroblast growth factor (bFGF, code ab16828, 1:200, Abcam, UK) were determined in human bone tissues and soft tissues. Deparaffinisation was carried out according to a conventional scheme. After rinsing (10 min.) in PBS (phosphate buffer, pH 7.4) solution, they were placed in a 4% hydrochloric sodium citrate buffer solution in a microwave oven for 20 minutes. After rinsing the cooled samples with PBS, each cut was covered with 150 fl of 3% hydrogen peroxide (10 min.). The samples were rinsed again in distilled water and in PBS, and then primary antibody (such as FGFR1, etc.) was dripped on the samples – 30 µl on each cut (exposure time – 2 hours), the samples were also covered with LSAB + LINK (with biotin linked secondary antibody) (code K1015, DakoCytomation, Denmark) for 30 minutes, and for 25 minutes they were covered with LSAB + KIT (streptavidin linked to the enzyme peroxidase) (code K0690, DakoCytomation, Denmark), and for 10 minutes – with DAB (chromogenic substrate system) (code K3468, Dako, Denmark). After that samples were briefly stained with hematoxylin. Summary information about all markers is in Table 5.2.3.2.The desired structures stained brown in case of a positive reaction.

Table 5.2.3.2.

Information about growth factors determined with biotin-streptavidin immunohistochemical method, growth factor receptors and tissue ECM degradation markers, gene proteins

Factor	**Source**	**Code**	**Working dilution**	**Producer and country**
TGFβ	mouse	1279	1: 1000	*Cambridge Science Park*, UK
bFGF	rabbit	ab16828	1:200	*Abcam*, UK
FGFR1	rabbit	ab10646	1:100	*Abcam*, UK
IL6	mouse	SC - 130326	1:50	*Santa Cruz*, USA
MMP8	mouse	SC - 80206	1:50	*Santa Cruz*, USA

(End of Table)

MMP9	rabbit	SC - 10737	1:250	*Santa Cruz*, USA
OPG	goat	SC - 8468	1:40	*Santa Cruz*, USA
OC	mouse	ab 13418	1:100	*Abcam*, UK
NkPb 105	rabbit	ab7971	1:100	*Abcam*, UK

5.3. TUNEL Method for Apoptosis Determination

The apoptosis set was used: *In Sit*u cell Death Detection, POD Cat.no.1684817, Roche Diagnostics (Germany) according to the technique described by Negoescu et al (1998). Deparaffinised tissue pieces were kept at room temperature in PBS (in phosphate buffer) (pH 7.5) solution for 10 minutes. After that blocking of endogenous peroxidase activity with 3% hydrogen peroxide was carried out for 30 minutes, then tissue pieces were washed (3 x 5 min.) in PBS solution. Pieces of tissue sections were placed in the citrate buffer solution and put in the microwave oven for 10 minutes to fix the antigen. Afterwards they were cooled to room temperature. Then they were washed in PBS solution and for 10 minutes placed in 0.1 % BSA (bovine serum albumin) on PBS. The tissue sections were incubated with TUNEL (terminal dezoxynucleotidyl transferase – mediated dUTP nick – end labeling mixture) at 37℃ in a container with humid air for 60 minutes. After rinsing, PBS tissue sections were incubated in POD (sheep antifluorescence antibodies coupled with horseradish peroxidase Fab fragment) at 37℃ for 30 minutes. Then the sections were covered with DAB (diaminobenzidine chromogen) for 7 minutes to determine peroxidase solution. Afterwards the tissue sections were rinsed in distilled water, hematoxylin and eosin were used for contact staining (for 20 sec.). The stained samples were processed with polystyrene and covered with a glass coverslip.

Histological sections were analysed with Leica DM RB light microscope at 400x magnification. To assess the apoptotic cell number, apoptotic cells were counted in 3 randomly selected visual fields that do not overlap each other in the histological sample (Itoh, 2007).

5.4. Semi-quantitative Data Conversion

To mark the relative frequency of growth factors, gene proteins, bone extracellular matrix proteins and degeneration enzymes, detected immunohistochemically, in the obtained tissue material the semi-quantitative counting method widely applied in the literature (Pilmane, 1998; Knabe, 2005) was used. The amount of growth factors, gene proteins, bone extracellular matrix proteins and degeneration enzymes was analysed in three visual fields of one section. The markings used are summarised in Table 5.4.3. To process the obtained data statistically, the number of cells observed in the visual field of the microscope was encoded (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field).

Table 5.4.3.

Markings of the relative frequency of the semi-quantitative method of growth factors, bone extracellular matrix proteins, degeneration enzymes and gene proteins, detected immunohistochemically

Used markings	**Explanations**	**Markings used in statistics**
0	No positive structures seen in the visual field	0
0/+	Occasional positive structures in the visual field	1

(End of Table)

+	Few positive structures in the visual field	2
+/++	Few to a moderate amount of positive structures in the visual field	3
++	A moderate amount of positive structures in the visual field	4
++/+++	A moderate amount to numerous positive structures in the visual field	5
+++	Abundant positive structures in the visual field	6

5.5. Data Processing Statistical Methods

The aim of the statistical data analysis was to evaluate with adequate statistical methods the expression of signalling molecules in patients of different age groups.

According to the variable type the central trend indicator was calculated – the arithmetic mean value, median and mode of the feature, as well as the distribution parameters – standard deviation, the minimum and maximum value of the feature.

To determine whether the data are consistent with normal distribution, the Shapiro-Wilk test was used.

To compare two dependent or independent groups according to one feature, the nonparametric Mann-Whitney test was used, but to compare several independent groups according to one feature – the nonparametric Kruskal-Wallis test was used.

In all cases the result was evaluated as statistically significantly different if the probability of the hypothesis was equal to 0.05 or less, i.e., the criterion for rejecting the null hypothesis was the significance level $\alpha = 0.05$. Otherwise, the null hypothesis was accepted.

For the analysis of two feature connection Spearman correlation analysis was used. The study adopted the following classification of correlation variables depending on the value of the correlation coefficient r:

- correlation is weak, if $r \leq 0,3$;
- correlation is moderate in cases, when $0,3 < r < 0,7$,
- but close correlation is, if $r \geq 0,7$.

The relationship between the two parameters was searched by using the linear regression method as well.

When analyzing nominal or ranking data, in order to compare two or more feature apportionment, Pearson's chi-square statistical analysis (if the contingency table frequency > 5) was used, while Fisher's exact test was used when the contingency table frequency < 5.

Statistical data processing was carried out using IBM SPSS Version 20.

6. RESULTS

6.1. Description of Patients

45 patients were included in the study, their mean age (±SD) was 22.02 ± 11.20. The minimum patients' age was 12 years, maximum – 49 years, age range 37 years. Age median was 17 years, but modal age – 12 years; interquartile age distribution was 15.5 years. Patient age asymmetry coefficient was 1.01, thus the distribution was with the right or positive asymmetry; age surplus (excess) coefficient was – 0.12. Using the Shapiro-Wilk test for age compliance with normal distribution, the test showed that the age of patients did not comply with normal distribution ($p < 0.001$). The histogram of all patients' age is represented in Figure 6.1.1.

The number of males involved in the study was 21 (46.67 %), while the number of females in the study was 24 (53.33 %), see Figure 1b; with the use of binominal test it was concluded that there was no statistically significant difference between the proportion of the male and female number ($p = 0.76$). Average male age (±SD) was 23.67 ± 11.02: minimum age 12 years, but maximum age 44 years. Average female age (±SD) was 20.58 ± 11.37: minimum age 12 years, but maximum age 49 years. Using the independent samples t test, it was concluded that the mean age difference of males and females participating in the study was 3.08 years [95% TI: – 9.84 to 3.67], and it is not statistically significantly different ($t(43) = 0.92$; $p = 0.36$). Based on the results of the Leuven test, it was found that the male and female age distribution dispersions were non-statistically significantly different ($F = 0.14$, $p = 0.70$).

Using the Shapiro-Wilk test to examine separately the compliance of male and female age distribution with normal distribution, it was found that they do not comply with normal distribution (respectively $p < 0.001$). The histogram of male and female age is represented in Figure 6.1.2.

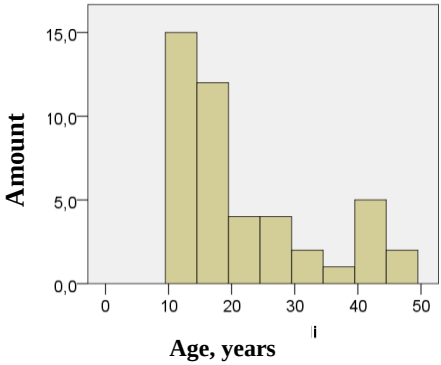

Figure 6.1.1. **The histogram of all patients' age**

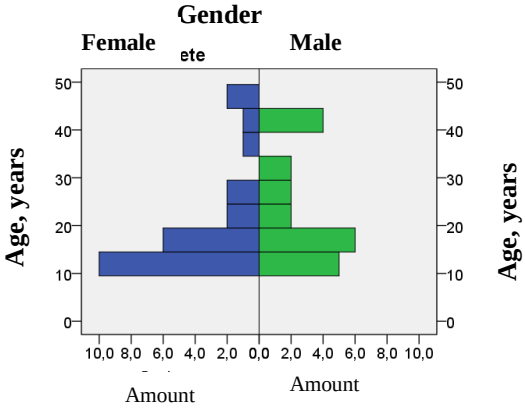

Figure 6.1.2. **The histogram of male and female age**

The percentage of male and female patients is shown in Figure 6.1.3. According to the percentage it can be seen that female patients were 53.33%, whereas male patients – 46.67%.

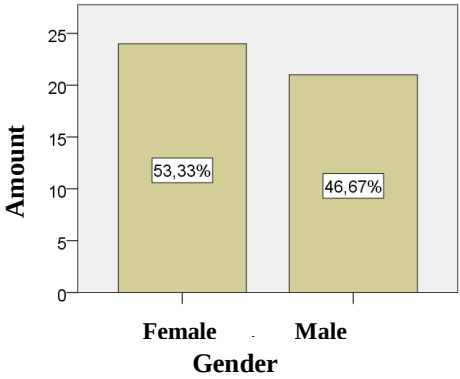

Figure 6.1.3. **The percentage of male and female patients involved in the study**

The number of patients involved in the study with orthodontic extraction was more than four times higher than the number of patients with irreversible pulpitis. See Figure. 6.1.4.

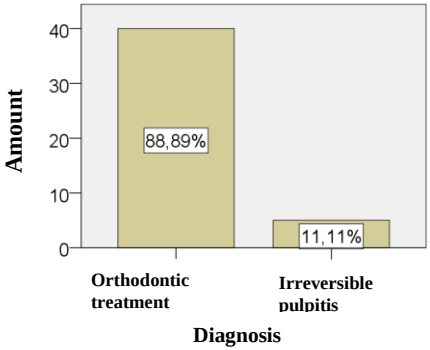

Figure 6.1.4. **The percentage of patients included in the study according to the diagnosis**

The number of patients included in the study with the diagnosis according to different age groups was as follows: in the 1st group all patients had tooth extractions due to orthodontic indications, 31.11% of the 2nd group patients had tooth extractions due to orthodontic indications, but in 2.22% cases due to the diagnosis of irreversible

pulpitis, the 3rd group patients in 24.44% cases had extractions due to orthodontic indications, but 8.89% of patients' teeth were extracted in the case of irreversible pulpitis. By studying the relationship between the age group and the diagnosis (Figure 6.1.5.), using Fisher's exact test, we concluded, that it existed and it was statistically significant ($p < 0.001$).

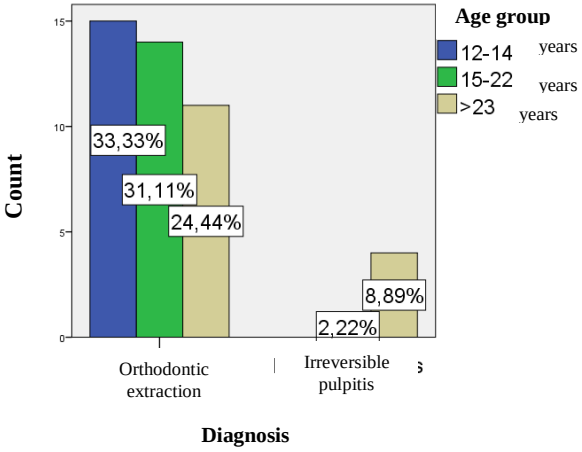

Figure 6.1.5. **The percentage of diagnosis according to different age of patients involved in the study**

6.2. Routine Histological Finding

While carrying out hematoxylin-eosin review staining, samples under analysis showed that the morphological picture of the 1st group patients (patients aged 12 to 14 years) corresponded to the generally accepted norm of growing bone tissues. Correct form osteocytes, osteon channels with a strong presence of blood vessels were found, but also there was presence of connective tissues in some osteon channels.

A similar picture to the 1st group was observed in the samples under analysis of the 2nd group patients (patients aged 15 to 22 years). However, the bone structure was more regular, corresponding to a mature bone.

The histologic picture, which was observed in the 3rd group patients (patients aged 23 years and over), corresponded to a mature bone structure with the signs of ageing, as well as with connective tissue proliferation in osteon channels.

6.3. Immunohistochemistry Findings: OPG

Analyzing the OPG expression in the interradicular septum we found that in the 1st group there was a differently numerous positive structures, but on average a moderate amount and numerous positive structures, while in the 3rd group few positive cells. On the basis of the Kruskal-Wallis test, we concluded that OPG for different age groups differ statistically significantly ($\chi 2 = 27.20$; df = 2; $p < 0.001$). OPG cell distribution by patients of different age groups is shown in Table 6.3.4.

Table 6.3.4.

Relative amount of OPG-contained cells in septum interradiculare of different age patient groups

Relative amount	12 − 14 years (n)	15 − 22 years (n)	23 − 49 years (n)
0	0	0	1
0/+	0	1	4
+	0	3	8
+/++	0	3	1
++	9	7	1
++/+++	2	1	0
+++	4	0	0

The relative amount of OPG positive structures in the 1st group was 4.8 ± 0.75, in the 2nd group it was 3.2 ± 0.8, in the 3rd group it was 1.5 ± 0.5. The median values of

OPG positive cells and 95% confidence interval for the patient groups are shown in Figure 6.3.6.

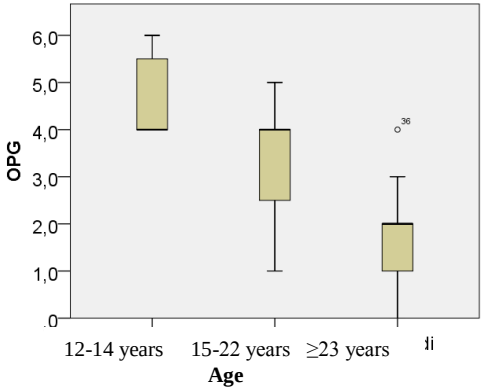

Figure 6.3.6. **Relative amount of osteoprotegerin positive cells in relation to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.3.1. Matrix Metalloproteinase 8 (MMP8)

Analyzing MMP8 expression in septum interradiculare tissues, we found that in the 1st group there was mostly few to a moderate amount of positive structures, in the 2nd group there were also mostly a moderate amount of positive structures, but also their absence, but in the 3rd group mostly few positive structures. On the basis of the Kruskal-Wallis test it was concluded that MMP8 for different age groups differs non-statistically significantly ($\chi 2 = 1.72$; df = 2; p = 0.42). Distribution of MMP8 positive structures in bone tissues by different age patient groups is shown in Table 6.3.1.5.

Table 6.3.1.5.

Relative amount of MMP8 positive structures in the interradicular septum tissues by different age patient groups

Relative amount	**12 − 14 years (n)**	**15 − 22 years (n)**	**23 − 49 years (n)**
0	0	0	2
0/+	3	3	0
+	1	4	7
+/++	4	5	2
++	6	3	1
++/+++	0	0	1
+++	1	0	2

The relative amount of MMP8 cells in the 1st group was 3.2 ± 0.6, in the 2nd group it was 2.5 ± 0.5, in the 3rd group it was 2.8 ± 0.75. The median values of MMP8 expression and 95% confidence intervals for patient groups are shown in Figure 6.3.1.7.

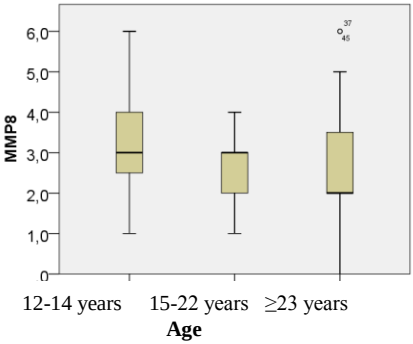

Figure 6.3.1.7. **Relation of MMP8 positive cell relative amount to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.3.2. Matrix Metalloproteinase 9 (MMP9)

When analyzing MMP9 expression in the interradicular septum tissues, we found that in the 1st group there was mostly a moderate amount of positive structures with some exceptions, in the 2nd group few to a moderate amount of positive cells , but in the 3rd group – mostly few positive cells. On the basis of the Kruskal-Wallis test it was concluded that MMP9 of different age groups differs non-statistically significantly ($\chi 2 = 0.72$; df = 2; p = 0.70). Distribution of MMP9 structures of patients by different age patient groups is shown in Table 6.3.2.6.

Table 6.3.2.6.

Relative amount of MMP9 positive structures in the interradicular septum tissues in different age patient groups

Relative amount	**12 − 14 years**	**15 − 22 years**	**23 − 49 years**
0	0	0	2
0/+	5	1	3
+	1	7	3
+/++	4	2	3
++	4	3	2
++/+++	1	1	1
+++	0	1	1

The relative amount of MMP9 cells in the 1st group was 2.9 ± 1.2, in the 2nd group it was 3.2 ± 1.1, in the 3rd group it was 2.2 ± 1.2. The median values of the relative amount of MMP9 positive structures and 95% confidence interval by patient groups is shown in Figure 6.3.2.8.

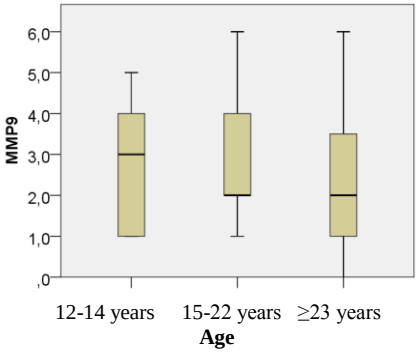

Figure 6.3.2.8. **Relation of MMP9 positive cell relative amount to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.3.3. Nuclear kappa Factor B105 (RANKL)

When analyzing RANKL expression in the interradicular septum tissues we found that in the 1st group there was a moderate amount to numerous positive structures, in the 2nd group there was mostly a moderate amount of positive structures, while in the 3rd group there were few positive cells. On the basis of the Kruskal-Wallis test, we concluded that RANKL for different age groups differ statistically significantly ($\chi 2$ = 14.70; df = 2; $p < 0.001$). The distribution of the relative amount of RANKL positive structures for patients of different age groups is shown in Table 6.3.3.7.

Table 6.3.3.7.

Distribution of relative amount of RANKL cells in the interradicular septum tissues in patients of different age groups

Relative amount	**12 − 14 years (n)**	**15 − 22 years (n)**	**23 − 49 years (n)**
0	-	-	-
0/+	0	2	3
+	1	1	8
+/++	0	2	0
++	9	8	4
++/+++	1	1	0
+++	3	1	0

The relative amount of RANKL positive cells in the 1st group was 4.5 ± 0.5, in the 2nd group it was 3.5 ± 0.6, in the 3rd group it was 2.5 ± 0.5. The median values of RANKL expression and 95% confidence interval in the patient groups are shown in Figure 6.3.3.9.

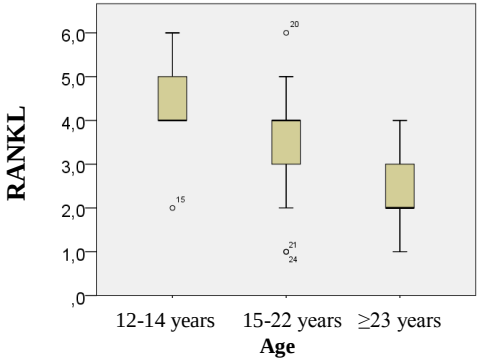

Figure 6.3.3.9. **Relation of relative amount of RANKL positive cells to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.3.4. Osteocalcin (OC)

When analysing the relative amount of osteocalcin cells in the interradicular septum tissues we found that in all groups, the 1st group, the 2nd group and the 3rd group there were mostly numerous to abundant positive structures.

The relative amount of osteocalcin cells in the 1st group was 6, in the 2nd group – 6, in the 3rd group – 6. The median values of positive osteocalcin structures and 95% confidence interval in the patient groups are shown in Figure 6.3.4.10.

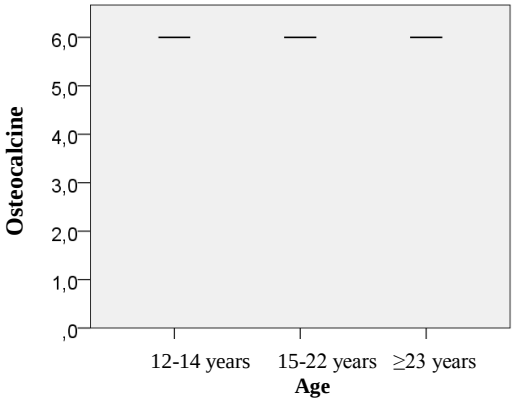

Figure 6.3.4.10. **Relation of relative amount of osteocalcin positive cells to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.3.5. Transforming growth factor β (TGFβ)

When analysing TGFβ expression in the interradicular septum tissues we found that in the 1st group there were mostly numerous to abundant positive structures, in the 2nd group there was mostly a moderate amount of positive structures, but in the 3rd group there was a moderate amount and also few positive structures. According to the Kruskal-Wallis test, we concluded that in different age groups TGFβ differs statistically significantly ($\chi 2 = 8.91$; df = 2; p = 0.01). The distribution of the relative amount of TGFβ cells in patients of different age groups is shown in Table 6.3.5.8.

Table 6.3.5.8.

Amount of TGFβ positive structures in septum interradiculare tissues in different age group patients

Relative amount	**12 – 14 years (n)**	**15 – 22 years (n)**	**23 – 49 years (n)**
0	-	-	-
0/+	-	-	-
+	2	1	2
+/++	0	2	4
++	3	5	9
++/+++	3	5	0
+++	5	0	0

The relative amount of TGFβ cells in the 1st group was 5.1 ± 0.9, in the 2nd group it was 4.5 ± 0.5, in the 3rd group it was 3.5 ± 0.5. The median values of TGFβ positive structures and 95% confidence interval in the patient groups are shown in Figure 6.3.5.11.

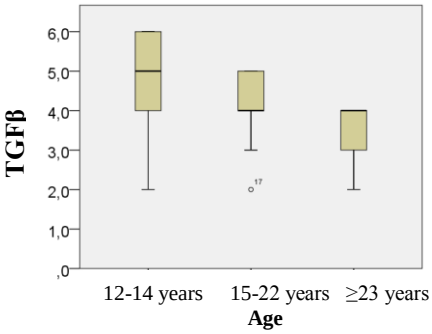

Figure 6.3.5.11. **Relation of the relative amount of TGFβ cells to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive

structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.3.6. Fibroblast growth factor receptor 1 (FGFR1)

When analysing FGFR1 expression in the interradicular septum tissues we found that in the 1st group there was a pronounced amount of positive structures, in the 2nd group there was a moderate amount of positive structures, and in the 3rd group there was a moderate amount of positive structures as well. On the basis of the Kruskal-Wallis test, it was concluded that FGFR1 in different age groups differs non-statistically significantly ($\chi 2=5.20$; df = 2; p = 0.07). The amount of FGFR1 positive structures in bone tissues in different age group patients is shown in Table 6.3.6.9.

Table 6.3.6.9.

Relative amount of FGFR1 positive structures in the interradicular septum tissues of different age patients

Relative amount	12 − 14 years (n)	15 − 22 years (n)	23 − 49 years (n)
0	-	-	-
0/+	0	1	0
+	2	0	3
+/++	1	1	3
++	2	5	3
++/+++	3	5	6
+++	7	2	0

The relative amount of FGFR1 positive structures in the 1st group was 4.9 ± 1.1, in the 2nd group it was 4.5 ± 0.5, in the 3rd group it was 4 ± 1.1. The median values of the amount of FGFR1 positive structures and 95% confidence interval in the patient groups are shown in Figure 6.3.6.12.

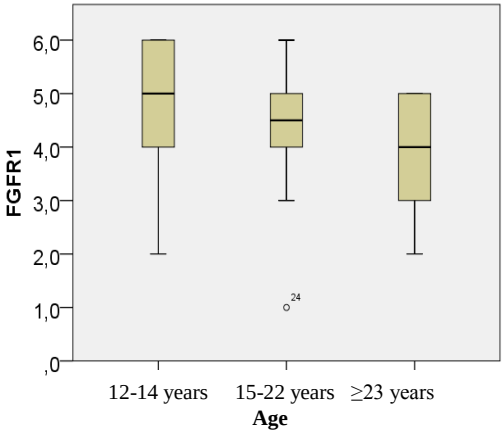

Figure 6.3.6.12. **Relation of the relative amount of FGFR1 structures to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.3.7. Base fibroblast growth factor (bFGF)

When analysing bFGF expression in the interradicular septum tissues we found that in the 1st group there were few to a moderate amount of positive structures, in the 2nd group there was a moderate amount of positive structures, and in the 3rd group there were mostly few positive structures. On the basis of the Kruskal-Wallis test, it was

concluded that bFGF in different age groups differs statistically significantly ($\chi 2 = 9.50$; df = 2; $p < 0.01$). The amount of bFGF positive structures in patients of different age groups is shown in Table 6.3.7.10.

Table 6.3.7.10.

Amount of bFGF positive structures in the interradicular septum tissues of different age group patients

Relative amount	**12 − 14 years (n)**	**15 − 22 years (n)**	**23 − 49 years (n)**
0	0	1	1
0/+	0	2	5
+	7	2	6
+/++	0	1	1
++	3	7	2
++/+++	2	2	0
+++	3	0	0

The relative amount of bFGF structures in the 1st group was 3.8 ± 1.2, in the 2nd group it was 3.1 ± 0.9, in the 3rd group it was 1.4 ± 0.6. The median values of the relative amount of bFGF positive structures and 95% confidence interval in the patient groups are shown in Figure 6.3.7.13.

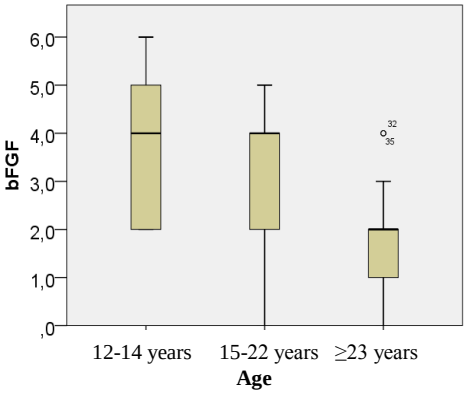

Figure 6.3.7.13. **Relation of the relative amount of bFGF positive structures to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.3.8. Interleukin 6 (IL6)

When analysing IL6 expression in interradicular septum tissues we found that in the 1st group there were few to a moderate amount of positive structures, in the 2nd group there were few to a moderate amount of positive structures with some exceptions, but in the 3rd group there was mostly a moderate amount of positive structures with some exceptions when there were few of them. On the basis of the Kruskal-Wallis test, it was concluded that the relative amount of IL6 for different age groups differs non-statistically significantly ($\chi 2=0.49$; df =2; p=0.78). The distribution of IL6 positive structures in bone tissues in different age group patients is shown in Table 6.3.8.11.

Table 6.3.8.11.

Distribution of IL6 relative amount in the interradicular septum tissues in different age group patients

Relative amount	**12 − 14 years (n)**	**15 − 22 years (n)**	**23 − 49 years (n)**
0	-	-	-
0/+	1	3	3
+	1	2	2
+/++	6	1	1
++	3	4	6
++/+++	1	3	1
+++	3	2	1

The relative amount of IL6 structures in the 1st group was 3.8 ± 0.7, in the 2nd group it was 4.1 ± 0.9, in the 3rd group it was 2.8 ± 1.2. The median values of IL6 expression and 95% confidence interval in the patient groups are shown in Figure 6.3.8.14.

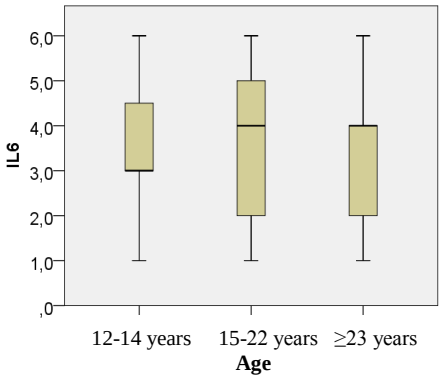

Figure 6.3.8.14. **Relation of the relative amount of IL6 positive structures to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.4. Characteristics of Apoptosis

When analysing IL6 expression in the interradicular septum tissues we found that in the 1st group there was abundant to a moderate amount of positive structures, in the 2nd group there was mostly a moderate amount of positive structures with exceptions between few and numerous, but in the 3rd group there were few to a moderate amount of positive structures. On the basis of the Kruskal-Wallis test, we concluded that the number of apoptotic cells for different age groups differs statistically significantly ($\chi 2$ = 10.82; df = 2; p = 0.004). The distribution of apoptosis cells in bone tissues in different age group patients is shown in Table 6.4.12.

Table 6.4.12.

Distribution of the relative amount of apoptotic cells in interradicular septum tissues in different age group patients

Relative amount	**12 − 14 years (n)**	**15 − 22 years (n)**	**23 − 49years (n)**
0	0	0	0
0/+	-	-	-
+	1	3	3
+/++	1	0	3
++	4	8	4
++/+++	-	-	-
+++	5	2	0

The relative amount of apoptosis cells in the 1st group was 5.1 ± 0.9, in the 2nd group it was 4.2 ± 1.75, in the 3rd group it was 3.1 ± 0.9. The median values of the relative amount of apoptosis cells and 95% confidence interval in the patient groups are shown in Figure 6.4.15.

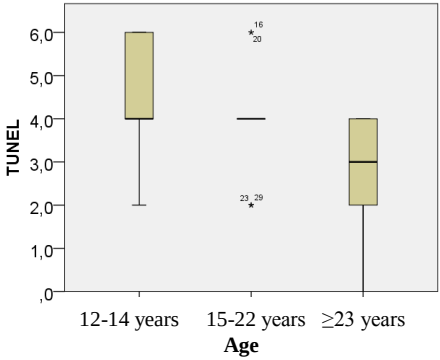

Figure 6.4.15. **Relation of the relative amount of apoptosis cells to patients' age** (0 – no positive structures seen in the visual field, (1) 0/+ – occasional positive structures in the visual field, (2) + – few positive structures in the visual field, (3) +/++ – few to a moderate amount of positive structures in the visual field, (4) ++ – a moderate amount of positive structures in the visual field, (5) ++/+++ – a moderate amount to numerous positive structures in the visual field, (6) +++ – abundant positive structures in the visual field)

6.5. Correlations between Diagnosis and bFGF

To compare average ranges of two independent samples, using the Mann-Whitney test, it was determined that only bFGF expression (Z=2.04; p=0.04) differs statistically significantly for both diagnosis, while the distribution of all the other factors in tissues differed non-statistically significantly (p>0.05).

The studied factors in relation to gender did not correlate and differed non-statistically significantly (p > 0.05).

6. 5. 1. Correlations of Different Factor Relative Amounts

The obtained immunohistochemical study results were analysed to determine cross-correlations (Table 6.5.1.13), and several interrelations of factors' relative amount were found.

Table 6.5.1.13.

Cross-correlation coefficients (rs) of different tissue factors and significance (relevance) levels (p) in tissues, where (*), if $p \leq 0.05$, (**), if $p \leq 0.001$

		OPG	MMP 8	MM P9	RAN KL	TGFβ	FGFR 1	bFGF	IL6
MMP8	r_s	0.14							
	p	0.35							
MMP9	r_s	0.03	0.27						
	p	0.79	0.06						
RANKL	r_s	0.66^{**}	0.21	0.23					
	p	0.00	0.16	0.13					
TGFβ	r_s	0.38^{*}	0.20	0.10	0.43^{**}				
	p	0.01	0.19	0.51	0.00				
FGFR1	r_s	0.25	0.142	0.20	0.37^{*}	0.62^{**}			
	p	0.09	0.35	0.18	0.01	0.00			
bFGF	r_s	0.27	0.32^{*}	0.27	0.31^{*}	0.48^{**}	0.41^{**}		
	p	0.06	0.02	0.06	0.03	0.00	0.00		
IL6	r_s	0.10	0.42^{**}	0.30^{*}	0.36^{*}	0.54^{**}	0.46^{**}	0.36^{*}	
	p	0.50	0.00	0.04	0.01	0.00	0.00	0.01	
TUNEL	r_s	0.42^{**}	0.00	0.33^{*}	0.46^{**}	0.21	0.36^{*}	0.10	0.19
	p	0.00	0.98	.045	0.00	0.20	0.03	0.53	0.26

Based on Spearman's correlation coefficient analysis, we found that, if OPG increases, the number of RANKL cells increases (Figure 6.5.1.16.), as both of these factors are linked by moderate and statistically significant correlation (r_s = 0.66; p < 0.001).

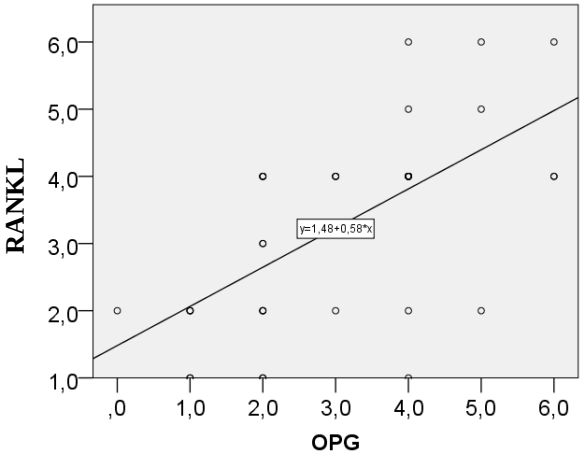

Figure 6.5.1.16. **Correlation between the relative amounts of OPG and RANKL**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amounts of OPG and TGFβ structures (r_s = 0.38; p = 0.01). Figure 6.5.1.17 shows the correlation between OPG and TGFβ.

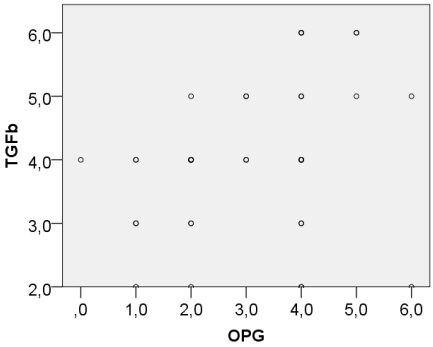

Figure 6.5.1.17. **Correlation between the relative amounts of OPG and TGFβ**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amounts of OPG and positive apoptosis cells ($r_s = 0.42$; $p < 0.001$). Figure 6.5.1.18 shows the correlation between the relative amounts of OPG and positive TUNEL structures.

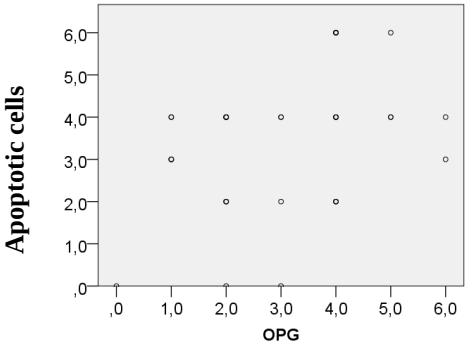

Figure 6.5.1.18. **Correlation between the relative amounts of OPG and apoptotic cells**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amounts of MMP8 and bFGF (r_s = 0.32; p = 0.02) (Figure 6.5.1 19).

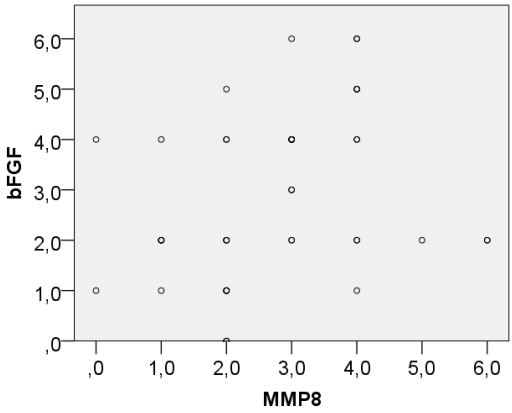

Figure 6.5.1.19. **Correlation between the relative cell amounts of MMP8 and bFGF**

Based on Spearman's correlation coefficient analysis, we found that there is a weak and statistically significant correlation between the relative amounts of MMP9 and IL6 (r_s = 0.30; p = 0.04) (Figure 6.5.1.20).

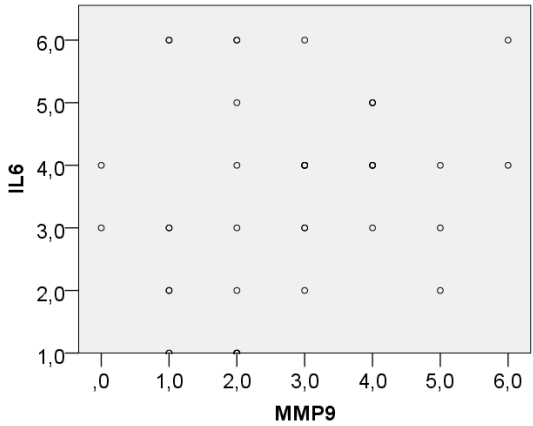

Figure 6.5.1.20. **Correlation between the relative cell amounts of MMP9 and IL6**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amount of MMP9 cells and the relative amount of apoptic cells ($r_s = 0.33$; $p = 0.04$) (Figure 6.5.1.21).

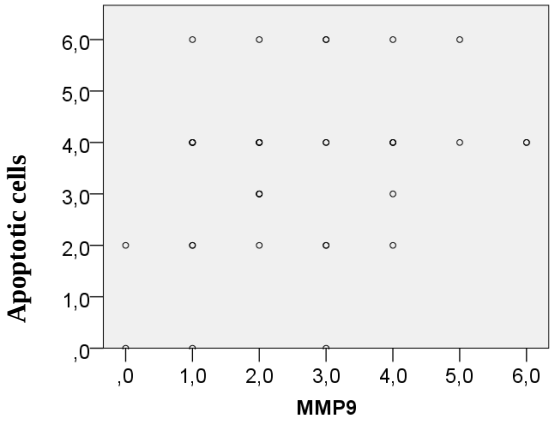

Figure 6.5.1.21. **Correlation between the relative amounts of MMP9 and apoptotic cells**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amounts of RANKL and TGFβ ($r_s = 0.43$; $p < 0.001$) (Figure 6.5.1.22).

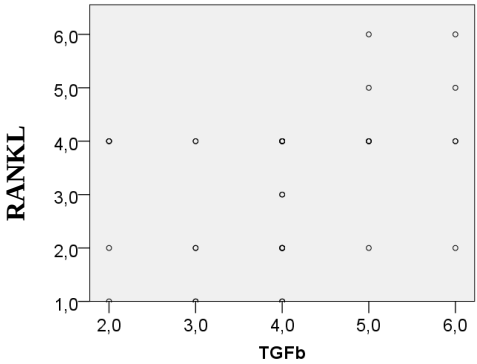

Figure 6.5.1.22. **Correlation between the relative cell amounts of RANKL and TGFβ**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amounts of RANKL and FGFR1 ($r_s = 0.37$; $p = 0.01$) (Figure 6.5.1.23).

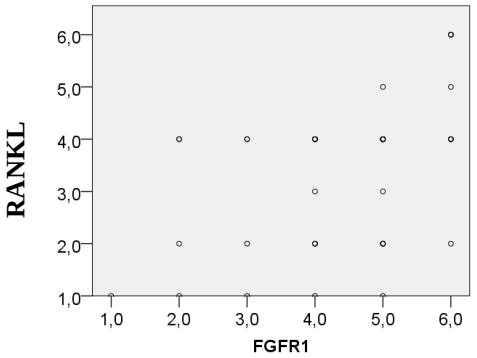

Figure 6.5.1.23. **Correlation between the relative amounts of RANKL and FGFR1**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amounts of RANKL and bFGF positive structures (r_s = 0.31; p = 0.03) (Figure 6.5.1.24).

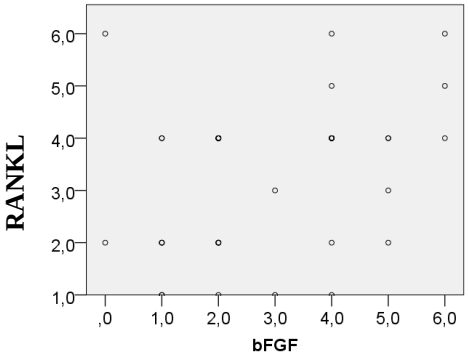

Figure 6.5.1.24. **Correlation between the relative cell amounts of RANKL and bFGF**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amounts of RANKL and IL6 (r_s = 0.36; p = 0.01) (Figure 6.5.1.25).

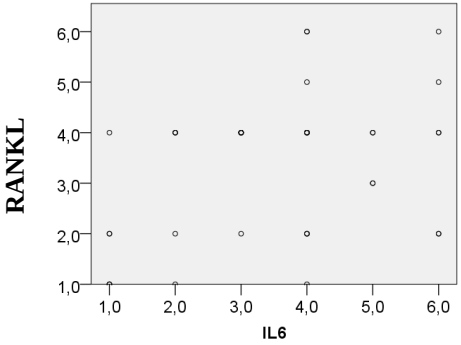

Figure 6.5.1.25. **Correlation between the relative cell amounts of RANKL and IL6**

Based on Spearman's correlation coefficient analysis, we found that there is a moderate and statistically significant correlation between the relative amounts of NKpB105 and apoptotic cells ($r_s = 0.42$; $p < 0.001$) (Figure 6.5.1.26).

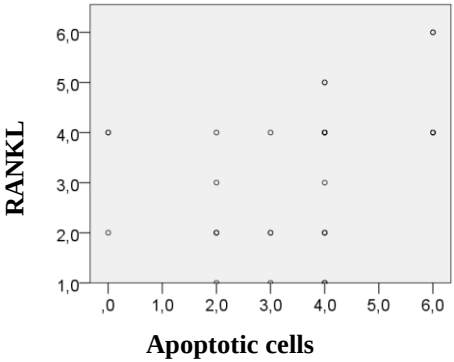

Figure 6.5.1.26. **Correlation between the relative amounts of RANKL and apoptotic cells**

7. DISCUSSION

Orthodontic studies that describe bone remodelling potential are usually linked with the already ongoing treatment. This is related to ethical aspects, which do not allow obtaining bone and PDS tissues from patients during the treatment process without any clinical justification, except in cases with combined orthognathic treatment.

Most of ethical reasons do not allow carrying out orthodontic studies using human tissue samples. However, when using animals in studies, it is not always possible to apply the results correctly to humans. Moreover, each person's individual characteristics and environmental impact can introduce corrections in the results. This is explained by constant human interaction with the environment, which is a combination of factors that under realistic conditions will cause treatment difficulties, in case the doctor has not considered them.

In our study a unique opportunity was used to obtain bone and PDS material before the foreseen orthodontic treatment, carried out in accordance with the treatment plan, in which tooth extraction for patients with major teeth compression or due to poor tooth prognosis was planned. The samples reflected the patients' bone and PDS conditions before any kind of treatment was applied; therefore, the results allow assessing the signalling molecule expression of tissue processes in a relative non-disturbed homeostasis condition. Determination of signalling molecule expression depending on age, thus, became a target helping to assess the processes that take place in bones and PDS. It is exactly the signalling molecule expression of tissue molecular processes that determines the treatment course, its pace and stability, which are important during the orthodontic treatment process.

Group distribution based on age is not accidental, but reflects the age of patients that is important for the bone and the surrounding tissue remodelling potential during orthodontic treatment, on which the chosen treatment tactics and restrictions depend (Proffit, 2008). The restrictions which are directly connected with the ontogenetic remodelling potential of the bone cause the main problems for orthodontic tooth movement and the final result of the treatment.

Patient gender distribution was almost equal, thus the signalling molecule expression does not differ by gender, which is also confirmed by the obtained results. Similar results were obtained according to the patient material type, where the major part was obtained after designated orthodontic extractions, while the proportion of extracted teeth due to irreversible pulpitis represented only the 10th part of all extractions.

The data of our study once again confirm the existing physiological bone remodelling guidelines, however, it provides a more detailed and individual picture of growth and remodelling factors, obtained from patients' interradicular septum tooth extraction places and compared with the available scientific literature.

7.1. Morphological Changes in Tissues

Analysing the bone tissue and soft tissue samples obtained in the study that were stained with haematoxylin-eosin, almost no changes that would differ from the generally accepted norm were detected in light microscopy. The exception was the proliferation of the Haversian canal connective tissues of the interradicular septum bone in some older patients, corresponding to the results by Ellen (1998) and Abiko (1998). Overall, morphological findings of the 1st and 2nd group patients corresponded to the norm for all study samples, without significant structural changes and with appropriate histology.

The interradicular septum bone tissues in the 3rd group patients were with uneven bone mineralization and we also found connective tissue proliferation in osteon canals. Sclerotization of some blood vessels in osteon canals was also detected. However, an adequate picture corresponding to norm was seen in around 30% of cases.

7.2. Growth Factors

There is little data in literature describing the distribution growth factors in human bone tissues in different age groups, but some literature sources and publications are available, where the distribution of similar growth factors is determined in various animal species.

In our study, the highest TGFβ1expression was observed in the 1st group, moderate – in the 2nd group patients, but for the 3rd group patients it was already decreased. This indicates that TGFβ1expression decreases depending on age. These results are also confirmed by Sanchez - Capelo (2005) study, in which the authors state that TGFβ1 helps to create a balance between cell growth and apoptosis. It has been observed that TGFβ1 can induce apoptosis in order to destroy immature cells. This corresponds to the assumption that in a growing bone and the surrounding structures as PDS, the mitotic activity is higher, which decreases with age.

Both Rosier (1998) and Oka (2007) in their studies indicate that TGFβ secretion is related to periosteal tissue proliferation. In addition, the most pronounced staining was observed in the cartilage cell growth and endochondral ossification zones, since TGFβ is secreted in places where active bone tissue regeneration occurs.

Verrecchia and Mauviel (2002) conclude that in the formation of extracellular matrix, TGFβ acts as a chemotaxis peptide directly affecting fibroblasts. TGFβ acts as an intermediary between ECM depleting proteases and their inhibitors, fully resulting in the creation of ECM forming proteins. Therefore, during active bone remodelling, the intensive expression of this factor promotes bone growth processes that are mostly observed in pubertal patients and the youth, which we observed in our patients as well. After active growth, these processes take place more slowly for the patients, thus TGFβ expression decreases as well.

In another study, Kloss and Gassner (2006), observing the patients aged 15 to 50 years, found a reduction in TGFβ expression depending on age, but related this finding to possible osteoporosis. Certainly, at large this cannot be attributed to the maxillofacial area, but it may be concluded that TGFβ expression is significant for bone homeostasis.

Tocharus (2004) obtained similar results in a study with mice, indicating that the decrease of TGFβ expression with age is associated with ECM protease activity required for tissue proliferation and differentiation.

Following Böttcher's (2005) findings, fibroblast growth factor (FGF) and its receptors (FGFR) are significant in different cellular processes, such as chemotaxis, cell migration, differentiation and apoptosis. In our study, we found a strong FGFR1 expression in the patients' bone tissues and the obtained results show that its expression is independent of patients' age. In contrast, bFGF expression correlates with patients' age, which allows concluding that stimulating growth factor receptor expression occurs relatively equally, with a slight prevalence in pubertal and maturation age, but growth factor expression decreases with age. Several authors report the importance of fibroblast growth factor in periodontal remodelling process, stimulating fibroblast proliferation (Ornitz, 2007; Mina, 2007). Therefore, it can be considered that the periodontal tissues of the patients involved in the study are characterized by sufficient regeneration potential, which is more expressed in the 1st and 2nd group patients.

In our study, FGFR1 expression in bone tissues from the interradicular septum was from a moderate amount to abundant structures in the visual field in all groups, but bFGF expression varied from a moderate amount to abundant in the 1st and 2nd groups, but to few and a moderate amount in the 3rd group. These results are indirectly confirmed by a study with rats by Naski (1998), where bFGF and FGFR1 expression was found in the youngest generation of the animal group in particular, compared to the already mature ones. The author concludes that the intensity of growth factor expression in the formation and maturation phase of the bone and surrounding tissues determines a more rapid tissue volume increase which contributes to further growth and formation. This suggests a better adaptation to remodelling processes particularly by the youth.

7.3. Bone Extracellular Matrix Proteins

Osteocalcin is a protein that is responsible for bone matrix mineralization. Osteocalcin expression in our patients showed a rich expression in the bone in all groups, regardless of patients' age, suggesting that mineralization occurs equally actively in all people under our study.

Osteocalcin is secreted by mature osteoblasts, odontoblasts and chondrocytes. Kloss et al. (2008) indicate that osteocalcin acts as a bone matrix signal and stimulates

human osteoclast migration and adhesion. Osteocalcin, which is a specific bone matrix protein, is significant in the process of resorption, and in the study with rats it was found that its activity reduces with age along with osteopontin expression, which was not established in our study. In a similar research, Ikeda (1996) explains the pronounced secretion of osteocalcin with the reduction of osteoblast activity and their reduced ability to respond to this signalling molecule stimulus. This partly explains the fact that bone loss risk increases with age, as osteoblastic activity, despite the presence of the signal, decreases and, thus, the ability to carry out an adequate bone remodelling process corresponding to the applied orthodontic force reduces as well. As a result, the bone loss is observed and the risk of gum recessions increases, which is rarely seen in younger patients.

7.4. Matrix Metalloproteinase

In our study, in tissue samples from the interradicular septum we found a moderate amount of MMP8 and MMP9 positive structures in all patient groups, which did not correlate with age. Pronounced MMP expression was observed only in some samples of the 3rd group, which could be explained by the ECM degrading processes that are more obvious in adult patients. It also corresponds to the study by Lamaitre et al. (2006), where greater MMP expression was observed particularly in adult patients. For the latter the ageing processes are accompanied by some pathophysiological reactions, such as the formation of osteoporosis.

MMP belongs to the degradation factors and is able to cleave a variety of extracellular matrix components (Verma, 2007). In addition, MMP participates in many biological processes, for example, angiogenesis, tissue regeneration and apoptosis (Lemaitre, 2006). Similar conclusions are drawn by Ikeda et al. (2001) in their study, when analysing MMP activity. The authors found that MMP expression and activity in adult tissues according to norm is relatively low, but it increases significantly in cases of different pathological process, which are caused by tissue destruction, such as inflammatory diseases, tumours and their metastases.

Manello (2006) in his work indicates that MMP and its inhibitors have a multipotent effect with a large range of activation, existing both during cell development time and their physiological activity. Moreover, these degrading enzymes provoke the expression of other molecules, related to bone and surrounding tissue development, maturation and remodelling. Inui et al. (1999) concluded that during osteogenesis, MMP is needed for ostoeclast migration to the bone surface, and MMP9 in particular participates in osteoblast activation during remodelling processes.

The results of our study also determined that MMP8 and MMP9 cell amount does not correlate, but there is a statistically significant correlation between MMP8 and IL6, as well as the relative amounts of MMP9 and IL6 positive structures. Together with degradation enzymes the latter functions as an inflammatory mediator. This shows that degradation enzymes are closely linked to the cytokine IL6 functions, namely, IL6 promotes the activation of other cytokines in the bone degradation process. This relationship appears also in Bourlier (2005) et al. in vitro study, in which the expression of signalling molecules is mutually dependent.

7.5. Cytokines

Statistically significant correlation was not found betweeen age and IL6 expression. IL6 was observed in a moderate amount of bone tissue cells in all age groups. The relative amount of IL6 correlated also with TGFβ, bFGF, FGFR1 and NkPb105, which confirms the significance of IL6 in physiological processes during remodelling processes, as well as during orthodontic treatment.

Similar conclusions draw also Alfons et al. (2004), who observed IL6 expression independently of age in their study with mice. The authors concluded that IL6 has high inflammatory mediator properties that affect both cartilage and bone remodelling capacity.

In another study Maugeri (2005) attaches a great importance to IL6 in osteogenesis, osteoclastogenesis and in bone remodelling processes. The author

concludes that the changes of IL6 expression and activation cause severe pathologies such as periodontitis, arthritis and osteoporosis.

Benatti et al. (2006) concluded in their study, that both IL6 and IL8 expressions are not related to ageing processes, but they are more affected by physiological and pathophysiological processes. In another study Benatti (2009) concluded that age does not affect IL6 and also other interleukin expression, but the changes of ageing affect cells, which has a negative impact on their ability to proliferate.

All the findings by the authors lead to a conclusion that IL6 participates in the majority of physiological processes and its activity range affects not only osteogenesis, but also other important functions of the organism. This is indicated by a large amount of other studies with interleukins that extends beyond maxillofacial area and covers the entire human body. Therefore, it is possible that IL6 has a multifunctional impact on all the tissues.

7.6. Functional Proteins of Bone Intercellular Space

Within our study NKPb105 protein which in literature is more known as RANKL (receptor activator nuclear kappa ligand) was also determined in the patients' interradicular septum tissue samples. The results indicate that RANKL expression reduces with age statistically significantly. In our data RANKL expression in the 1st group was detected in numerous cells, in the 2nd group in a moderate amount of cells and in the 3rd group from few to a moderate amount was in the cells.

Similarly as RANKL, also OPG expression reduced with age statistically significantly. What differed was the relative amount of its structures, in the 1st group there were abundant cells, in the 2nd group in a moderate amount of cells and in the 3rd group it was in few cells.

Such results may explain why larger bone loss occurs with age, and both bone regeneration and remodelling potential reduce. With a reduced OPG expression and with

a prevailing RANKL expression, stimulation of osteoclastic activity occurs, when a bone degrading cell activity results in a decreased bone volume, density and structure.

Since RANK/RANKL system and OPG are competing signalling molecules, it could be expected that the 1st and 3rd group patients should have inversely proportional RANKL and OPG expressions, indicated by Cao (2003) in his *in vitro* and *in vivo* study with mice. Having compared our results with the above mentioned study, we noted a difference, i.e., we did not observe an inversely proportional decrease in RANKL and OPG expressions in the human bone tissue samples. This could be explained by the fact that the study by Cao was carried out with animals and it cannot be directly interpreted into human material. In his discussion the author indicates that the results obtained in cell cultures are open to criticism as the cell age cannot be accurately determined and, therefore, to apply these results to humans to a full extent would not be correct.

In her article that summarizes several data of RANK/RANKL studies, Narducci (2011) highlights the importance of this signalling molecule in the whole process of bone remodelling. Approximately 10% of the bone amount of the organism is remodelled every year. This process is regulated by the cells that act as antagonists and also RANKL and OPG are antagonists by their nature. Therefore, it is impossible to treat these molecules separately.

As it is indicated by the author, the expression of these molecules is dependant on age, but their proportion changes depending on the remodelling process, i.e., whether it is physiological or pathological. Therefore, the authors' data also confirm our findings that the relative amount of OPG is affected by age.

Hofbauer et al. (2008) in his study notes that the most significant changes related to the ageing process cause the development of osteoporosis. It affects the bone features dramatically.

If RANKL and OPG proportion affects younger patients, then, as indicates Rouster-Stevens (2007) in his study, juvenile idiopathic arthritis develops most

frequently. In the study the author admits that the changes in the operational system of these molecules cause irreversible effects on bone tissues.

Venuraju (2010) study, which is linked more to cardiology, indicates that increased OPG distribution affects coronary vascular disease development, since OPG expression stimulates increased coronary calcification.

Kosteniuk (2005) in his study with rabbits found the reduction of OPG and RANKL expression depending on age. As the author concludes, the expression decrease of these signalling molecules is directly related to age, as, with ageing, the ability of cells to express the required quantity of this substance decreases.

7.7. Apoptosis in Bone Tissues and PDS

According to Bodine (2008), at the end of the remodelling cycle mature osteoblasts have three different further development paths. They can either become osteocytes in a mineralized bone matrix, or become the cells placed on the external surface of a growing bone in a single layer and protect the bone matrix from osteoclasts, but ¾ of the cells become apoptotic. Balanced functioning of all these processes ensures bone remodelling and reconstruction. It has been found that the main regulators of bone metabolism, such as TGFβ, RANKL and OPG, as well as other growth factors and cytokines, integrins, estrogens, glucocorticoids and parathyroid hormone, by modulating the apoptosis process, regulate life span of osteoblasts and osteocytes (Chan, 2002).

In our study the amount of apoptotic cells in the interradicular septum decreased with patients' age. This shows that the bone and periodontal tissue remodelling is slower with age. It is possible that in the 1st group bone rebuilding takes place faster and more intensively, but with the increase of patients' age, especially in adult patients in the 3rd group the process of apoptosis does not take as long. Together with the remodelling process more intensive cell migration and physiological action take place, which explains the prevalence of cells affected by apoptosis. This is particularly well observed in bone tissues, since osteocyte and osteoblast life span compared to other cells in the body, is much longer. Osteocytes can reach even 10 years (Mescher, 2013). In his study

Weinstein (2003) relates the latter observation with the amount of osteoblasts – the greater the amount of osteoblasts in a particular sample, the greater the chance to detect apoptotic cells. For instance, there are about 20-50 osteoblasts in a human biopsy material, but in a rodent bone there are 800-1200 osteoblasts. When analysing the correlations of our results, we found that there is a statistically significant, close correlation between OPG, RANKL and FGFR1 and the amount of apoptotic cells – with the increase of OPG, RANKL and FGFR1 expression, the amount of apoptotic cells increases in the tissues from the interradicular septum. This is an indication of increased cell proliferation necessary to ensure active growth and continuously active remodelling process. Our finding is also confirmed by the study summary carried out by Xing (2005), as well as the study by Khosla (2002), who indicate the correlation of RANKL and apoptosis.

It is relevant to note that both RANKL and OPG regulate osteoclast proliferation, differentiation and maturation, therefore, they are naturally linked with apoptotic processes occurring in the bone formation and remodelling process, which, apparently, is topical also for the patients under our study from the orthogenetic aspect.

8. SUMMARY

Growth factors and their receptors important in bone morphogenesis (TGFβ, FGFR1), bone extracellular matrix protein (OC), degeneration enzymes (MMP8, MMP9), bone intercellular space functional proteins (RANKL, OPG) and apoptosis indices show varying results in bone tissues and in PDS depending on patients' age

Depending on age, uneven mineralization, vascular sclerosis and connective tissue proliferation in osteon canals can be observed in the bone tissues of the 3rd group patients. In contrast, morphological findings in younger patients correspond to the histological norm for all the investigated samples.

Our study results show that with the increase of patients' age a decrease of some amount of signalling molecule expression occurs, such as OPG, RANKL, TGFβ, and apoptosis decreases with age as well. The largest amount of apoptotic cells was found in the 1st group where patients in the age of puberty were included, which is consistent with the general concept of bone tissue remodelling.

Mutual MMP8, MMP9 and IL6 correlation is explained by IL6 range of activity, which includes many functional processes that occur physiologically in the cell activity and also in relation to pathological phenomena which we have not studied in our work. Interleukin activity is extensive and further in-depth studies are needed for it.

Overall the results of the study show that there is a multiplicity of tissue remodelling potential peculiarities depending on the patient's age. The aims drawn forward by our hypothesis have been reached that, undoubtedly, proves the complicated mechanism of human ageing. It includes everything that has been described and the interaction of many other factors as well, resulting in significant effect on cell functioning and the ability to adjust to external factors. The mechanical force as an external factor determines the outcome and prognosis of the orthodontic treatment. Immunohistochemical studies would help to go deeper into the biomechanical processes and explain them, which, basically for orthodontists will be helpful to estimate the duration of treatment.

9. CONCLUSIONS

1. The ageing process of interradicular septum bone tissues does not correlate with gender, but correlates with ageing itself. The overall morphological picture in the intact interradicular septum ageing bone tissues includes uneven bone tissue mineralization, blood vessel sclerotisation and connective tissue proliferation in osteon canals.

2. With age TGFβ decreases in the interradicular septum bone tissues statistically significantly, suggesting the decrease of active bone growth induction and reduction of bone remodelling through the weakening of the programmed cell death mechanism.

3. Bone tissues of interradicular septum and periodontal ligaments are characterised by intensive FGFR1 secretion unrelated to age, indicating steady-inducing effect of FGFR1 in any tissues of mesoderm origin. Reduction of bFGF from the ontogenetic aspect in the interradicular septum tissues substantiates the reduction of bone tissue metabolic processes independent of Type 1 receptors.

4. Marked, unrelated to age, OC secretion in the interradicular septum bone tissues justifies both the mineralization of bone tissues and the basestock protein of these bones as the marker of non-specific tissue changes.

5. MMP8 and MMP9 secretion is not dependant on interradicular septum ageing, but on bone growth processes and other cytokine secretion stimulation, which is proved by statistically significant correlation of both enzymes with IL6.

6. Significant IL6 secretion in the interradicular septum tissues of patients of all ages indicates the multifunctional nature of this cytokine and the link with local bone remodelling processes.

7. Statistically significant decrease of OPG and RANKL with the patient's age proves the decrease of bone tissue cell functional activity from the ontological aspect simultaneously with the reduction of cell proliferation.

8. Statistically significant decrease of apoptotic cells in the interradicular septum of human bone tissues with age points to the decrease of bone remodelling. Mutual correlation of OPG, RANKL, TGFβ and apoptosis indicates the overall reduction in the interradicular septum cell activity, osteoclastic activity, bone growth and remodelling potential from the ontogenetic aspect.

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11. PUBLICATIONS AND PRESENTATIONS ON THE STUDY THEME

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2. Maris Grzibovskis, Ilga Urtane, Mara Pilmane. Signalling molecule expression assessment in alveolar septum in different age groups. 7th Congress of the Baltic Orthodontic Association. Kaunas, Lithuania, 2011, thesis and oral presentation. P. 27.

3. Maris Grzibovskis, Mara Pilmane, Ilga Urtane. Specific signalling molecule expression in periodontal ligaments in different age groups. Baltic Morphology VII Scientific Conference, Riga, 2013. P.57.

Local thesis and presentations – (7)

1. Maris Grzibovskis, Ilga Urtane, Mara Pilmane. Quantitative assessment methodology of specific signaling molecules in alveolar bone in different age groups. Book: Annual RSU Scientific Conference 2010 in the Field of Medicine. Riga, 2010, Thesis and presentation.

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