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Morphological Changes in Bone and Cartilage Tissue Affected by Facial Clefts and During Tissue Regeneration

Summary of the Doctoral Thesis for obtaining the scientific degree "Doctor of Science (*Ph. D.*)"

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The Doctoral Thesis is available the RSU Library and on the RSU website: https://www.rsu.en/dissertations

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Abbreviations used in the Thesis

AAI	Institute of Anatomy and Anthropology
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BMP-2/4	bone morphogenetic protein 2/4
ECM	extracellular matrix
HBD	human beta defensin
HBD-2	human beta defensin 2
HBD-3	human beta defensin 3
IL-10	interleukin 10
IL-1α	interleukin 1 alpha
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IMH	immunohistochemistry
MMP	matrix metalloproteinase
MMP-2	matrix metalloproteinase 2
MMP-8	matrix metalloproteinase 8
MMP-9	matrix metalloproteinase 9
OA	osteoarthritis
OC	osteocalcin
OPG	osteoprotegerin
OPN	osteopontin
RANKL	nuclear kappa factor B ligand
Rs	*Spearman'*s rank correlation coefficient
RSU	Rīga Stradiņš University
Runx2	runt related transcription factor 2
TGFβ	transforming growth factor beta

TGFβ1	transforming growth factor beta 1
TIMP	matrix metalloproteinase tissue inhibitor
TIMP-2	matrix metalloproteinase tissue inhibitor 2
TNF-α	tumor necrosis factor alpha
TUNEL	deoxynucleotidyltransferase-mediated DNA 3'-tip labeling with deoxyuridinphosphate
VEGF	vascular endothelial growth factor
Wnt	*Wingless-type* MMTV integration site protein
Wnt3a	*Wingless-type* MMTV integration site protein 3a

Introduction

Facial development begins during the 4th embryonic week when five facial folds are formed around the primary oral cavity and surround it: the forehead and nose fold, the fold of the maxillary pair, and the fold of the lower jaw pair (Bhat, 2020). During embryogenesis, nerve ridge cells migrate to the orofacial region, where they contribute to the formation of bones, vertebrae, and other connective tissues of the face (Rezzoug et al., 2011). A set of congenital facial defects formed as a result of incomplete growth of facial folds is called facial clefts, to which the alveolar and palate cleft also belong. Facial clefts are a clinical defect that affects speech formation, respiratory function through the nose, auditory development, olfactory function, and aesthetics (Nasreddine et al., 2021). In the world, facial clefts are found in one per 600–800 newborns, thus they are one of the most common congenital cranoifacial pathologies (Vyas et al., 2020). In Latvia, on average, one child with a cleft is born per 700–800 newborns (Akota et al., 2001).

Correction of facial clefts occurs through surgical intervention and usually requires more than one surgery to restore normal physiological functions, especially in cases where it has affected the supporting tissues (Ha et al., 2015).

The structure of the facial skeleton consists of two types of tissue – cartilage tissue and bone tissue, the components of which are chondroblasts and osteoblasts, which are formed from one ancestor (Hartmann, 2006), as well as the development of facial clefts, is influenced by a number of signalling factors, cytokines, tissue proteinases and their inhibitors, growth factors, gene proteins, tissue proliferation factors and apoptosis, the role of which in the development of facial clefts in the world is still at the research stage. It is important to understand which tissue factors and signalling molecule regulation pathways could affect the regeneration of tissues and their successful healing after surgery.

Mineralisation factors, such as osteocalcin (OC), osteopontin (OPN), and osteoclast genesis suppressant – osteoprotegerin (OPG), are found in the bone and are proteins responsible for various tissue functions. They play a role in the process of mineralisation of bone tissue, its regulation, as well as in various anti-inflammatory processes (Bai et al., 2014; Lund et al., 2009), however, their significance in relation to repeated surgeries of patients with facial clefts has not been fully explored.

More and more attention is paid to various growth factors: bone morphogenetic protein (BMP-2/4), transforming growth factor beta 1 (TGFβ1), and basic fibroblast growth factor (bFGF), in connection with their ability to influence the outcome of wound healing, as well as their need for normal palate development (Nakajima et al., 2014; Zhou et al., 2019). Studies in mice have shown that the inactivation of BMP receptors in the mesenchyma of the maxillary bone and the oral epithelium can lead to cleft lip and palate (Li et al., 2013), however, further research is needed on the effect of these factors on the bone and cartilage tissues affected during surgery.

Tissue proteinases and their inhibitors play an important role in tissue remodeling during craniofacial development (Yoon et al., 2003). Matrix metalloproteinases (MMPs), which are subdivided into several subunits, such as matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 8 (MMP-8) and matrix metalloproteinase 9 (MMP-9) cleave tissue and extracellular matrix (ECM) (Cowan et al., 2009; Yu et al., 2019). Matrix metalloproteinase tissue inhibitors (TIMP) have the ability to inhibit MMP activity. Altered MMP to TIMP ratios have been found in tissues affected by cleft lip and palate (Verstappen and Von den Hoff, 2006).

Cytokines, such as interleukin 1 alpha (IL-1α) and interleukin 10 (IL-10), when interacting with tissue protective factors – human beta defensins (HBD), regulate inflammatory processes. IL-1α acts as an inflammatory mediator and

increases the formation of osteoclasts, their differentiation and activity in the bone (Ji et al., 2002). IL-10 is a cytokine that regulates inflammatory reactions by reducing the synthesis of anti-inflammatory cytokines and chemokines (Zhang et al., 2014).

HBDs are small cationic peptides with a broad spectrum of antimicrobial effects, especially human beta defensin 2 (HBD-2) and human beta defensin 3 (HBD-3), which can promote bone remodeling by reducing the risk of bacterial contamination and subsequent immune response of the body (Warnke et al., 2013). These factors have been extensively studied, however, there is a lack of studies that show their interactions with each other in cleft-affected tissues.

Gene proteins are necessary in the morphogenesis of facial structures and their role in the development of facial clefts is being studied. The Runt-related transcription factor 2 (Runx2) gene protein is a transcription factor necessary for the correct arrangement of osteoblasts (Komori, 2010). The gene protein signalling path of the Wingless type of the MMTV integration site (Wnt) family plays a regulatory role in facial morphogenesis, since its ligand gene secretion is found in the tissues of the facial folds, and its changes are associated with the development of facial clefts (Jin et al., 2012).

Apoptosis, or programmed cell death, characterises the process of cell death. Programmed cell death plays a role in the development of the palate, however, the molecular mechanisms that control apoptosis of the oral periderm have not yet been fully studied (Li et al., 2017). The great importance for apoptosis could be not only in the process of formation and fusion of the embryonic palate, but also in the postnatal period after cleft correction, and successful tissue adhesion.

However, despite the general functions of various factors, there are no studies in the world on tissue factors/genes that look at changes in various tissue

factors in patients affected by facial clefts after the first surgery on bone and cartilage in facial cleft tissue and after repeated cleft surgery.

Aim of the Thesis

To determine the distribution and relative number of different tissue factors (mineralisation factors, growth factors, remodeling factors, protective factors, cytokines, gene proteins, and apoptosis) of cleft affected tissue (cartilage and bone tissue) immunohistochemically after the first time and in repeated surgeries.

Tasks the of Thesis

1. To determine the relative number and distribution of tissue proteinases (MMP-2, MMP-8, MMP-9) and their inhibitors (TIMP-2), mineralisation factors (OC, OPN), osteoclast genesis suppressor (OPG), growth factors (TGFβ1, BMP-2/4, bFGF), cytokines (IL-1α), tissue protective factors (HBD-2, HBD-3, IL-10), gene proteins (Runx2, Wnt3a) and apoptotic cells in healthy tissues – bone and cartilage tissue.
2. To determine the relative number and distribution of all the above-mentioned factors (MMP-2, MMP-8, MMP-9, TIMP-2, OC, OPN, OPG, TGFβ1, BMP-2/4, bFGF, IL-1α, HBD-2, HBD-3, IL-10, Runx2, Wnt3a, and apoptotic cells) in the cleft bone and cartilage tissues operated on for the first time.
3. To determine the relative number and distribution of all the above factors in re-operated cleft bone and cartilage tissue.
4. To compare and find statistically significant differences of the data for the bone and cartilage tissue of the control patients, first-time operated and re-operated cleft patients.

5. To determine the correlation of all the above factors in the bone and cartilage tissue of the control patients, first-time operated and re-operated cleft patients.

Hypothesis of the Thesis

1. The relative quantity and distribution of tissue markers differ between the bone and cartilage tissue of patients affected by facial clefts and those of control patients.
2. The relative number and distribution of markers in the bone and cartilage tissue of first-time operated and re-operated cleft patients is qualitatively different.

Novelty of the Thesis

Although individual studies on the factors affecting the development of facial clefts are carried out worldwide, there is practically no data on the primary morphological changes of the cleft bone and cartilage tissue (alveolar bone, nasal septum, vomer) and those in the course of tissue regeneration after various cleft correction operations due to the complex collection of the material, the long-term collection and also for ethical reasons.

For the first time, complex studies of tissue markers in bone and cartilage tissues were conducted in the group cleft patients operated on for the first time, and in the group of re-operated patients, and the relative number and distribution of various tissue markers in cleft-affected bone and cartilage tissues were determined and compared with the same tissue markers in healthy tissues.

1 Materials and methods

1.1 Material used for the morphological study and the characterization of patient groups

The bone and cartilage tissue material for the morphological study was collected by the Institute of Stomatology of Rīga Stradiņš University (RSU) at the Cleft Lip and Palate Centre, in the period from 2003 to 2018. During osteoplasty, the bone tissue affected by the clefts of the alveolar process of the maxillary bone was obtained. During nasal correction, or rhinoplasty, the bone tissue affected by clefts was obtained from the anterior nasal spine or vomer of the alveolar bone, as well as hyaline cartilage tissue from clefts in the nasal septum or nasal wing. The research material was prepared in the RSU AAI Morphology Laboratory for routine histological and immunohistochemical methods.

49 patients were included in the study: 17 patients had a bilateral cleft of the lip, alveolar growth and palate, 31 patients had a unilateral cleft of the lip, alveolar growth and palate. One patient had a unlateral partial cleft lip and palate. The total number of morphologic bone material obtained from patients who underwent osteoplasty or rhinoplasty for the first time was 14. The number of morphologic bone material from reoperated patients was 22. The number of morphologic cartilage material from the bone and cartilage tissue surgeries in which rhinoplasty was performed as the first surgery, was 17. The number of cartilage tissue samples obtained from rhinoplasty, performed as repeated surgery was 21.

Patients were divided into four groups according to the type of bone and cartilage tissue and the order of surgery, and two control groups were formed:

Group 1 – bone control group, from which bone tissue was obtained during operations that were not associated with clefts.

Group 2 – cartilage control group, from which cartilage tissue was obtained during operations not associated with clefts.

Group 3 – patients with cleft of lip, alveolar growth and palate, who underwent surgery on the bone tissue of the maxillary bone for the first time.

Group 4 – patients with cleft of lip, alveolar growth and palate, who have undergone surgery on the cartilage tissue of the nasal correction for the first time.

Group 5 – patients with cleft of lip, alveolar growth and palate with bone tissue samples, who have undergone repeated surgery on the bone and cartilage tissue of the cleft.

Group 6 – patients with cleft of lip, alveolar growth and palate with cartilage tissue samples, who have undergone repeated surgery on the bone and cartilage tissue of the cleft.

1.2 Morphological methods

1.2.1 Fixation of the tissue material

Fixation of tissue samples in Stefanini's solution (Stefanini et al., 1967), which lasted 24 hours, was performed at the RSU Institute of Stomatology's Cleft Lip and Palate Centre immediately after rhinoplasty and osteoplasty. The following reagents were required to prepare the tissue fixation solution: 150 ml picric acid, 20 g paraformaldehyde, 425 ml Sorensen buffer (pH 7.2) and 425 ml distilled water. After fixation, the tissue material to be examined was transported to the Morphology Laboratory of the Institute of Anatomy and Anthropology of Rīga Stradiņš University for further processing. The fixed tissue material was rinsed in a thyroid buffer with 10 % sucrose for 24 hours. Next, the tissue material was then dehydrated with alcohol liquids of different concentrations: alcohol 50°, 70°, 90°, 95° and finally with absolute alcohol. Then the tissue material was degreased in xylol for 30 minutes and stored for one hour in paraffin I and two hours in paraffin II. The paraffin with a dispenser was

poured into special cassettes to obtain paraffin blocks. From these, 3 μm thin tissue sections were prepared using a semi-automatic rotary microtome (*Leica RM2245, Leica Biosystems Richmond Inc.,* United States). They were then transferred to slides (*HistoBond®+, Paul Marienfeld GmbH & Co. KG,* Germany) and placed in a thermostat at 56 ºC for 20–60 minutes to dry. The tissues were then deparrffinised in xylol, dehydrated with alcohol of various concentrations and prepared for immunohistochemistry and TUNEL methods.

1.2.2 Routine histology staining method

Routine staining of bone and cartilage tissue with hematoxylin and eosin was performed to obtain a review morphological overview (Fischer et al., 2008). Tissue sections were dried in a thermostat, deparaffinised in xylol and dehydrated with alcohols of different concentrations (alcohol I 96º for 3 minutes; alcohol II 96º 3 minutes; alcohol III for 70º 3 minutes), then stained with hematoxylin (ab143166, *Abcam,* United States) and eosin (code 05-B10003, *Eosin Y alcoholic solution, Bio Optica Milano S.p.A.,* Italy). Tissues were then rinsed again under running water, dehydrated with an alcohol solution of increasing concentration (alcohol for 70° – 3 minutes, alcohol for 96° – 3 minutes and alcohol for 96° another 3 minutes) and covered with carboxylene and xylene. Histological glue was then applied (code-H875.2; *Carl Roth GmbH + Co,* Germany) and micropreparations were covered with a slide. The final shaded slides were analysed under a light microscope (*Leica DM500RB, Leica Biosystems Richmond Inc*., United States).

1.2.3 Immunohistochemistry method and reagents

The following factors were determined in bone and cartilage tissue using the immunohistochemical method of biotin and streptavidin (Hsu et al., 1981):

- **matrix metalloproteinase 2** (MMP-2, code orb101049, derived from rabbit, polyclonal, working dilution 1:400, Biorbyt, United States);

- **matrix metalloproteinase 8** (MMP-8, code orb18114, derived from rabbit, polyclonal, working dilution 1:100, Biorbyt, United States);
- **matrix metalloproteinase 9** (MMP-9, code orb18114, derived from rabbit, monoclonal, EP1254, working dilution 1:100, Biorbyt, United States);
- **matrix metalloproteinase tissue inhibitor 2** (TIMP-2, code orb18114, derived from rabbit, monoclonal, 3A4, working dilution 1:100, Biorbyt, United States);
- **bone morphogenetic protein 2/4** (BMP-2/4, code AF355, derived from goat, polyclonal, working dilution 1:100, R&D Systems, Germany);
- **basic fibroblast growth factor** (bFGF, code ab16828, derived from rabbit, polyclonal, working dilution 1:200, Abcam, UK);
- **transforming growth factor beta 1** (TGFβ1, code orb7087, derived from rabbit, polyclonal, working dilution 1:200, Biorbyt, United States);
- **interleukin 1 alpha** (IL-1α, code orb308787, derived from mouse, polyclonal, working dilution 1:100 p.m., Biorbyt, United States);
- **interleukin 10** (IL-10, code orb100193, derived from rabbit, polyclonal, working dilution 1:600, Biorbyt, United States);
- **human beta defensin 2** (HBD-2, code sc-20789, derived from rabbit, polyclonal, working dilution 1:100, Biorbyt, United States of America);
- **human beta defensin 3** (HBD-3, code orb183268, derived from rabbit, polyclonal, working dilution 1:100, Biorbyt, United States of America);
- **osteopontin** (OPN, code orb11191, derived from rabbit, polyclonal, working dilution 1:100, Biorbyt, United States);

- **osteoprotegerin** (OPG, code orb11189, derived from rabbit, polyclonal, working dilution 1:100, The Orbit, United States);
- **osteocalcin** (OC, code orb259644, derived from rabbit, polyclonal, working dilution 1:100, Biorbyt, United States);
- **runt related transcription factor 2** (Runx2, code ab192256, derived from rabbit, monoclonal, ERP14334, working dilution 1:250, Abcam, UK);
- **wingless type MMTV integration site 3a protein** (Wnt3a, code ab1992, derived from rabbit, polyclonal, working dilution 1:800, Abcam, UK).

The tissue sections were dewaxed twice in xylol (xylol I – 5 minutes; xylol II – 5 minutes) and dehydrated with alcohols of different concentrations (alcohol I 96º – 3 minutes, alcohol II 96º – 3 minutes; alcohol III 70º – 3 minutes). The tissues were then rinsed twice within 5 minutes with a THREE buffer solution (code 15-M106, *Bio-Optica*, Italy) and boiled twice in a microwave in an EDTA buffer (code T0103, *Diapath*, Italy). Tissue samples were then covered with 3 % peroxide for 10 minutes to block endogenous percosidase and rinsed twice with THREE buffer solution for 5 minutes. After that, the tissues were incubated with primary antibodies for one hour. Information on all antibodies used in immunohistochemistry is summarised in Table 2.8. *Antobody Dilutent* (code ab6422, *Abcam*, United States) was used to dilute all antibodies.

For antibodies of mouse and rabbit origin, *the HiDef DetectionTM HRP Polymer* (code 954D-30, *Cell MarqueTM,* United States) system was used. After incubation and rinsing the primary antibodies in TRIS buffer three times within 5 minutes, *the HiDef DetectionTM* reaction amplifier (code 954D-31, *Cell MarqueTM,* United States) was applied for 10 minutes. Next, the preparations were rinsed repeatedly in the TRIS buffer three times for 5 minutes and the *HiDef DetectionTM HRP polymer* marker (code 954D-32, *Cell MarqueTM,* United

States) was applied for 10 minutes. Finally, samples were re-rinsed in the TRIS buffer 3 times over 5 minutes, coated with *a DAB Substrate Kit* color imaging system (code 957D-60, *Cell MarqueTM,* United States) and incubated for 10 minutes to visualise the positive structures in brown.

For the BMP-2/4 antibody produced from the goat, the *ImmunoCruzTM ABC* (code sc-2023, *Santa Cruz Biotechnology, Inc*., United States) staining system was used. Micropreparations were incubated with 1.5 % blocking serum in TRIS buffer for up to one hour at room temperature. Then the preparations were incubated with the primary antibody for one hour at room temperature and re-rinsed with a TRIS washing buffer three times within 5 minutes. Next, they were incubated with a biotin-containing secondary antibody (biotinised goat immunoglobulin) for 30 minutes and rinsed in TRIS buffer solution three times within 5 minutes. Then the peroxidase complex of avidin and biotin horseradish was added, and the preparations were incubated for 30 minutes at room temperature and rinsed three times within 5 minutes with TRIS buffer solution. Finally, the tissue preparations were coated with *the DAB Substrate Kit* coloring system and incubated for 10 minutes to visualise the positive structures in brown.

After incubation with chromogen, the tissue samples were rinsed under running water and contrast stained with cell nuclear dye – hematoxylin (code 05-M06002, *Mayer's Hematoxylin, Bio Optica Milano S.p.A*., Italy). The micropreparations were then rinsed in distilled water and dehydrated in spirit of increasing concentration, cleared in xylol and coated with histological glue and a cover glass. Negative controls were also made for tissue sections in which the primary antibody was replaced with *Antibody Dilutent*, and positive controls were performed with tissues in which a positive reaction has always been shown. The final preparations were analysed under a light microscope (*Leica DM500RB, Leica Biosystems Richmond Inc*., United States), processed with *the Image Pro*

Plus program and photographed with a digital camera (*Leica DC 300F, Leica Microsystem AG,* Germany).

1.2.4 TUNEL method

Apoptosis, or programmed cell death in tissues, was determined by deoxynucleotidyltransferase dUTP-labeled end marking, or TUNEL method, described in 1996 by Negoescu (Negoescu et al., 1996). A standardised set of TUNEL kit (code 13799800, 1:10*, In Situ Cell Death Detection Kit, Roche Diagnostics*, Germany) was used to evaluate apoptosis. Initially, tissue preparations were deparaffinised with xylol and solutions of decreasing ethanol concentration (99 % ethanol, ethanol 95 % and 70 % ethanol). They were then rinsed in distilled water and TRIS solution for approximately 10 minutes. Subsequently, the endogenous peroxidase was blocked for 10 minutes with 3 % hydrogen peroxide and tissue preparations were rinsed in TRIS solution three times within 5 minutes. The tissue preparations were then placed in EDTA buffer for 10 minutes and placed in the microwave for 5 minutes, which were then cooled to room temperature to be rinsed again in TRIS solution and placed in 0.1 % cow serum albumin phosphate buffer. The tissue preparations were then incubated with theTUNEL mix (*TUNEL Enzyme solution*) for one hour at 37 °C. They were then rinsed again in THREE buffer solution and incubated twice within 5 minutes with *Converte-POD solution* for 30 minutes at 37 °C. The samples were then rinsed in a TRIS buffer and coated for seven minutes with *a DAB* (diaminebenzidine chromogen) solution for peroxidase detection and rinsed again with distilled water. In the next step, contrast staining with hematoxylin was performed, polystyrene was incorporated and covered with a cover glass. The finished tissue preparations were also analysed with a light microscope (*Leica DM500RB, Leica Biosystems Richmond Inc.,* United States), processed with *the Image Pro Plus program* and photographed with a digital camera (*Leica DC 300F, Leica Microsystem AG,* Germany).

1.2.5 Semi-quantitative counting method

The relative amount of immunohistochemically positive osteocytes and chondrocytes was evaluated according to a semi-quantitative counting method (Pilmane et al., 1998):

- 0 – no positive structure was detected in the visual field;
- 0/+ – a rare occurrence of positive structures in the visual field;
- + – a few positive structures in the visual field;
- +/++ –few to moderate number of positive structures in the visual field
- ++ – a moderate number of positive structures in the visual field
- ++/+++ – moderate to numerous positive structures in the visual field
- +++ – numerous positive structures in the visual field
- +++/++++ – numerous to abundant number of positive structures in the visual field
- ++++ – abundant number of positive structures in the visual field

The relative amount of positive structures was analysed in five randomly selected fields of view for each tissue incision preparation. For further analysis of positive structures, the average amount of positive structures was applied.

1.2.6 Statistical methods

The statistical analysis of all data from the study was conducted with *Statistical Package for the Social Sciences* (SPSS) version 23 (IBM *Corporations*, United States). Descriptive statistical methods were used to process the research data. Nonparametric tests were used in the study as the data sample did not match the normal distribution. Differences between control and study group data were analysed using the Mann-Whitney U test (Riffenburgh, 2012a). Spearman's rank sequence correlation coefficient (r_s) was used to evaluate the interrelationships between variables, which is derived from the ratio of the difference in rank pairs to the number of observations (Riffenburgh,

2012b). For the data statistics for processing, the results of the semi-quantitative method were converted into numerical values. The data conversion scheme is described in Table 2.10. The correlations obtained were ranked as follows: 0–0.350 was classified as weak correlation, 0.351–0.700 as moderately strong, 0.701–1 as strong. The results were considered statistically significant if the p-value was < 0.05 (Andrade, 2019).

2 Results

2.1 Morphological findings in control and cleft patients

Routine Histology

The bone tissue was subjected to routine histologic analysis, in which the changes in bone coloration, osteons, and infiltration of the Haversian canal were examined. Cartilage tissue was assessed by perichondrium, cell zones and matrix staining.

In the patients in the bone control group, the morphological structure of the bone corresponded to the general norm. Osteocytes with a preserved number of osteons were visualised and no pronounced proliferation of connective tissue was observed in the canals.

In the patients in the cartilage control group, the morphological structure of the cartilage corresponded to the general norm. In all cases, the cartilage areas were clearly distinguished, as well as most patients had a more pronounced area of cartilage growth with a greater number of chondrocytes, while in the area of mature cells the number of chondrocytes was lower. In most cases, the intermediate area between the groups of isogenic chondrocytes became homogeneous.

Osteons of various sizes were observed in the bone tissue of cleft-affected patients, their Haversian canals were filled with connective tissue, and the bone plates were heterogeneous in coloring and irregular in size.

In the cartilage tissue of patients affected by clefts, all areas of cartilage were observed. In most patients, the area of mature cells where chondrocytes were placed in a group of isogenous cells, predominantly 2–4 cells, was more pronounced. In general, most cartilage samples visually corresponded to the generally accepted norm.

2.2 Findings of immunohistochemically determined factors and apoptosis

2.2.1 OPN

OPN was not detected in all bone tissue in patients in the control group. In general, rare (0/+) OPN positive osteocytes were observed in bone tissue in the control group.

OPN containing osteocytes were observed in all bone tissue of cleft patients undergoing surgery for the first time. Their numbers varied from rare (0/+) to moderate to numerous (++/+++). In general, there were detected a few (+) OPN positive osteocytes in the bone tissue of cleft patients undergoing surgery for the first time (see Figure 2.1).

OPN was observed in all bone tissue of patients with repeated surgery. Their numbers varied from rare (0/+) to numerous (+++). Overall, there were few OPN positive osteocytes (+) in the bone tissue of patients with repeated surgery on cleft bone and cartilage tissue (+).

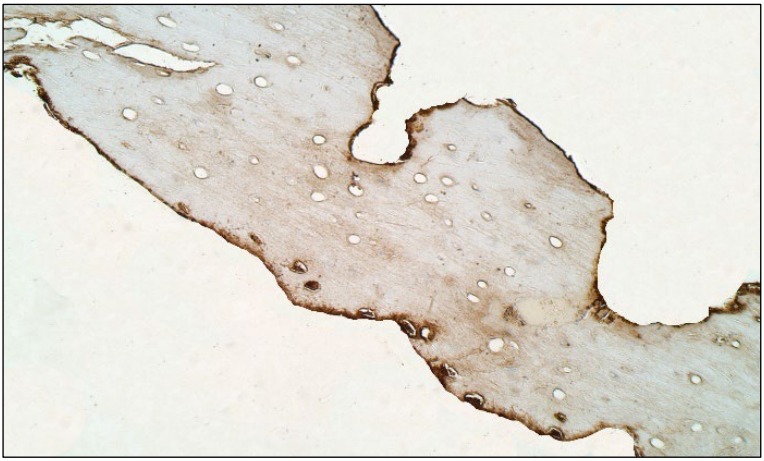

Figure 2.1 **Rare (0/+) OPN positive osteocytes in the bone tissue of 8 years and 5 months old cleft patient undergoing the first surgery**
OPN IMH, × 200

Statistical data. In the case of this factor, no statistically significant difference was found between the bone tissue of cleft patients undergoing surgery for the first time and the bone tissue of cleft patients that had been re-operated. **A statistically significant difference was found both between the control group and the first–time operated cleft patient group** ($U = 21.00$; $p = 0.028$) **and between the reoperated cleft patient group and the control group** ($U = 34.50$; $p = 0.025$). This meant that there were significantly more OPN positive osteocytes in the bone tissue of cleft-affected patients after both the first and repeated surgeries than in the control group.

The presence of OPN was observed in all cartilage tissue of patients in the control group. The number of positive chondrocytes varied from few (+) to numerous to abundant (+++/++++). Overall, the control group's cartilage tissue showed moderate number of OPN positive chondrocytes (++).

OPN was detected in all cartilage tissue of cleft patients undergoing surgery for the first time. It numbers ranged from numerous (++) to abundant (++++). In general, for the patients undergoing first surgery, OPN positive chondrocytes were observed to be numerous to abundant (+++/+++++) (see Figure 2.2).

The presence of OPN was observed in all cartilage cells of reoperated cleft patients. The number of OPN positive chondrocytes varied from moderate (++) to abundant (++++). In general, in the cartilage tissue of reoperated cleft patients, OPN positive chondrocytes were observed to be numerous (+++).

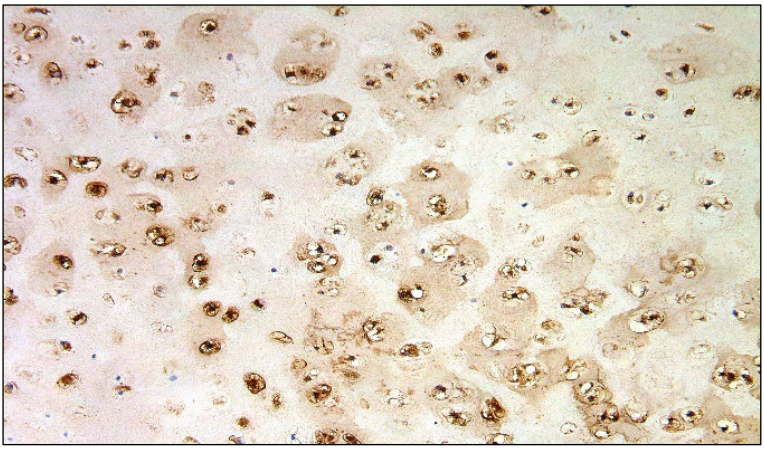

Figure 2.2 **Numerous to abundant (+++/++++) OPN positive chondrocytes of 12 years and 8-month old cleft patient undergoing the first surgery**
OPN IMH, × 400

Statistical data. In the case of OPN, no statistically significant difference was found between the cartilage tissue of cleft patients undergoing surgery for the first time and the cartilage tissue of re-operated cleft patients. **A statistically significant difference was found both between the control group and the first-time operated cleft patient group** ($U = 22.50$; $p < 0.001$) **and between the reoperatedly operated cleft patient group and the control group** ($U = 25.50$; $p < 0.001$). This meant that there were significantly more OPN positive chondrocytes in the cartilage tissue of cleft-affected patients after both the first and repeated surgeries than in the control group.

2.2.2 OC

OC was observed in all bone tissue in patients in the control group. Its results ranged from moderate to numerous (++/+++) to abundant (++++) OC positive osteocytes in the field of vision. In general, numerous OC positive osteocytes were observed in the bone tissue of patients in the control group (+++).

The presence of OC was detected in all bone tissue of cleft patients undergoing surgery for the first time. It ranged from rare (0/+) to moderate to numerous (++/+++). In general, for the patients undergoing first-time surgery, OC positive osteocytes were observed few to moderate (+/++) (see Figure 2.3).

OC was found in all bone tissue of reoperated cleft patients. Its numbers varied from rare (0/+) to numerous (+++). In general, in the bone tissue of reoperated cleft patients, OC positive osteocytes were found to be few to moderate to moderate (+/++ to ++).

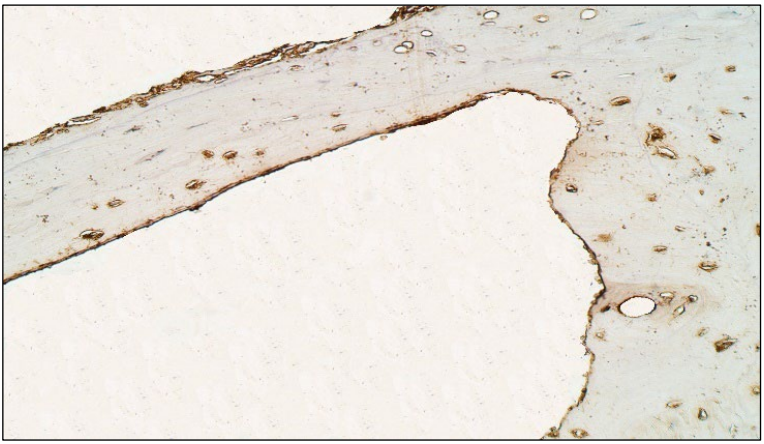

Figure 2.3 **Few to moderate (+/++) OC positive osteocytes in the bone tissue of a 14 years and 5months old first-time operated cleft patient**
OC IMH, × 200

Statistics. No statistically significant difference in the relative number of OC positive osteocytes was found between first-time operated cleft patients' bone tissue and re-operated cleft patients' bone tissue. However, there was a statistically significant difference between the control group and the two groups of cleft-affected patients, where **cleft bone tissue after the first surgery**

($U = 1.00$; $p < 0.001$) **and recurrent surgery** ($U = 14.00$; $p = 0.001$) **presented a lower number of OC positive osteocytes compared to the control group**.

OC was detected in all cartilage tissue of patients in the control group. Its numbers varied from moderate (++) to numerous to abundant (+++/++++). In general, OC-positive chondrocytes were found to be numerous (+++) in the control group's cartilage tissue.

OC was found in all cartilage tissue of first-time operated patients. It ranged from numerous (++) to abundant (++++) (see Figure 2.4). In general, there were observed numerous (+++) OC positive chondrocytes in the cartilage tissue of patients undergoing surgery for the first time.

OC was found in all cartilage tissue of reoperated cleft patients. It ranged from moderate (++) to abundant (++++). In general, OC positive chondrocytes were found to be numerous (+++) in the cartilage tissue of re-operated cleft patients.

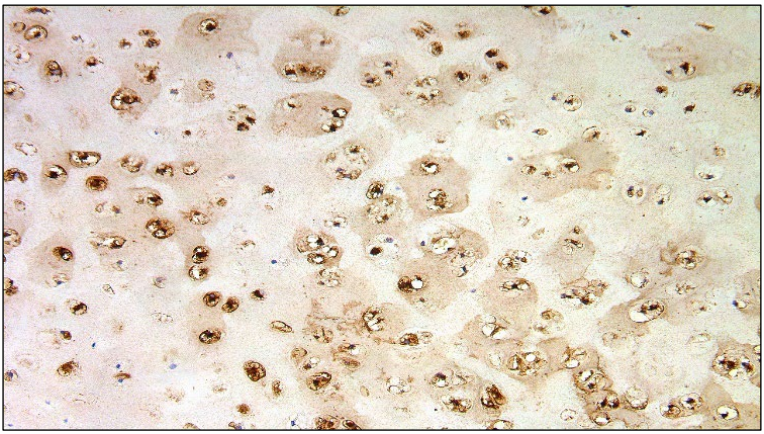

Figure 2.4 **Moderate to numerous (++/+++) OC positive chondrocytes in 8 years and 3 months old cleft patient undergoing first-time surgery**
OC IMH, × 200

Statistical data. When evaluating the mean ranks of the relative numbers of immunohistochemically detected OC positive chondrocytes, no statistically significant difference was observed between the study and control groups.

2.2.3 OPG

OPG was present in all bone tissue of patients in the control group. The amount of OPG in osteocytes varied from rare (0/+) to numerous (+++). In general, rare (0/+) OPG positive osteocytes were detected in the bone tissue of patients in the control group.

OPG positive osteocytes were observed in all bone tissue of cleft patients undergoing surgery for the first time. The number of osteocytes containing OPG in the field of vision varied from rare (0/+) to numerous (+++) (see Figure 2.5). In general, there were observed few (+) OPG positive osteocytes in the first-time operated cleft patients.

The presence of OPG was observed in all bone tissue of reoperated cleft patients. Its results ranged from rare (0/+) to numerous (+++). In general, there were observed few (+) OPG positive osteocytes in the bone tissue of reoperated cleft patients.

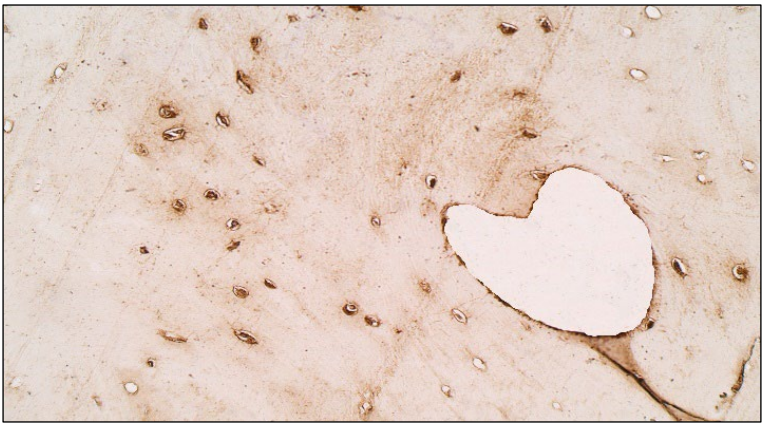

Figure 2.5 **Moderate (++) number of OPG positive osteocytes in 8 years and 1 month old cleft patient undergoing first surgery**
OPG IMH, × 200

Statistical data. No statistically significant difference was found between OPG positive osteocytes in the bone tissue of first-time operated cleft patients and in re-operated cleft patients' bone tissue. Nor was there a statistically significant difference found between the control group and the cleft patients.

The presence of OPG was observed in all cartilage tissue of patients in the control group. It ranged from moderate to numerous (++/+++) to abundant (++++). In general, OPG positive chondrocytes were observed numerous to abundant (+++/++++) in the control group's cartilage tissue.

The presence of OPG was observed in all cartilage cells of patients undergoing surgery for the first time. Its numbers varied from few (+) to abundant (++++) (see Figure 2.6). In general, numerous OPG positive chondrocytes were observed (+++) in the cartilage tissue of patients undergoing surgery for the first time.

The presence of OPG was observed in all cartilage tissue of reoperated cleft patients. Its numbers varied from few (+) to abundant (++++). In general,

numerous OPG positive chondrocytes (+++) were observed in the cartilage tissue of reoperated cleft patients.

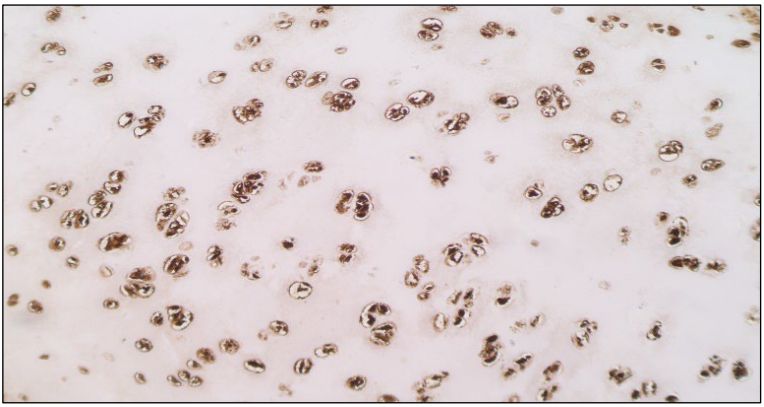

Figure 2.6 **Numerous to abundant (+++/++++) OPG positive chondrocytes in the cartilage tissue of 7 years and 7 months old first-time operated cleft patient**
OPG IMH, × 200

Statistical data. It was found that the relative amount of mean immunohistochemically determined OPG positive chondrocytes in cartilage tissue did not differ statistically significantly between the study and control groups.

2.2.4 BMP-2/4

The presence of BMP-2/4 was observed in all bone tissues of patients in the control group. It ranged from few (+) to moderate to numerous (++/+++). In general, few to moderate BMP-2/4 positive osteocytes were observed in the control group (+/++).

BMP-2/4 positive osteocytes were observed in all bone tissue of first-time operated cleft patients. The amount of osteocytes containing BMP-2/4 in the field of view varied from rare (0/+) to moderate (++) (see Figure 2.7). In general,

BMP-2/4 positive osteocytes were observed from numbers rare to few (0/+ to +) in cleft patients undergoing surgery for the first time.

The presence of BMP-2/4 was observed in all bone tissue of reoperated cleft patients. The amount of BMP-2/4 positive osteocytes varied from rare (0/+) to numerous (+++). In general, there were few to few to moderate (+ to +/++) BMP-2/4 positive osteocytes found in bone tissue of reoperated cleft patients.

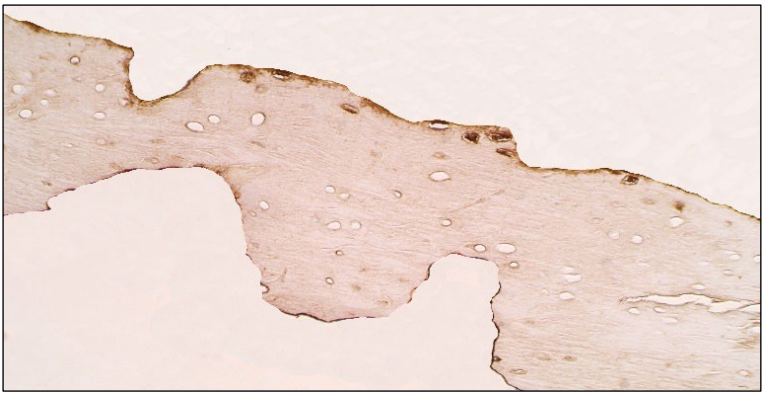

Figure 2.7 **Rare (0/+) BMP-2/4 positive osteocytes in the bone tissue of an 8 years and 11 months old first-time operated cleft patient**
BMP-2/4 IMH, × 200

Statistical data. In the case of this factor, no statistically significant difference was found between the bone tissue of first-time and re-operated cleft patients. With Mann–Whitney U test **a statistically significant difference between the control group and the first-time operated cleft patient group** ($U = 22.00$; $p = 0.036$), was obtained, where fewer BMP-2/4 positive osteocytes were observed in cleft patients than in the control group.

The presence of BMP-2/4 was detected in all cartilage tissue of patients in the control group. Its results ranged from few to moderate (+/++) to numerous (+++). In general, BMP-2/4 positive chondrocytes were observed in moderate numbers (++) in the cartilage tissue of the control group.

BMP-2/4 was found in all cartilage tissue of patients undergoing surgery for the first time. It ranged from few to moderate (+/++) to abundant (++++) (see Figure 2.8). In general, BMP-2/4 positive chondrocytes were in numerous (+++) amount in the cartilage tissue of first-time patients.

The presence of BMP-2/4 was detected in all cartilage tissue of reoperated cleft patients. Its results varied from few (+) to abundant (++++). In general, BMP-2/4 positive chondrocytes were found to be numerous (+++) in the cartilage tissue of reoperated cleft patients.

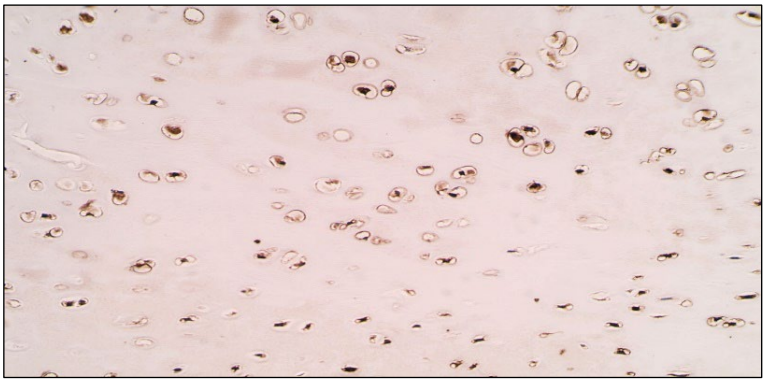

Figure 2.8 **Moderate number (++) of BMP-2/4 positive chondrocytes in 11 years and 9 months old cleft patient cartilage tissue undergoing first-time surgery**
BMP-2/4 IMH, × 200

Statistical data. **A statistically significant difference was found between the control group and the reoperated cleft patient group**. This means that BMP-2/4 positive chondrocytes were present in the overwhelming majority in cartilage tissue of reoperated cleft patients ($U = 60.50$; $p = 0.025$) compared to the control group. There was no statistically significant difference between the other groups with the Mann–Whitney U test.

2.2.5 bFGF

The bFGF was detected in all bone tissue of patients in the control group. Its amount in osteocytes varied from few (+) to numerous (+++). In general, there were few (+) bFGF positive osteocytes observed in bone tissue in the control group.

The bFGF was detected in all bone tissue of cleft patients undergoing surgery for the first time. Its presence varied from rare (0/+) to moderate (++). In general, the bFGF positive osteocytes were rarely (0/+) observed in bone tissue in cleft patients undergoing surgery for the first time (see Figure 2.9).

The bFGF was present in all bone tissue of reoperated cleft patients. Its presence varied from rare (0/+) to numerous (+++). In general, the bFGF positive osteocytes were rare (0/+) in the bone tissue of patients with re-operated cleft.

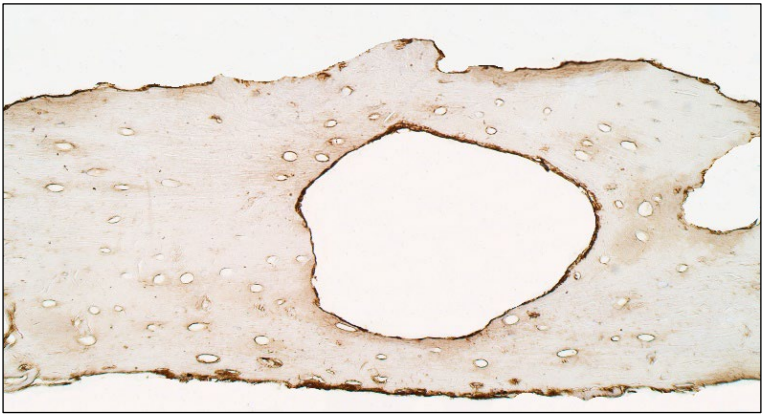

Figure 2.9 **Rare (0/+) bFGF positive osteocytes in the bone tissue of a cleft patient undergoing the first surgery at 8 years and 11 months of age**
bFGF IMH, × 200

Statistical data. The mean relative number of immunohistochemically determined bFGF positive osteocytes did not differ statistically significantly between the groups of operated cleft patients. **A statistically significant**

difference was observed between the first-time operated cleft patients and the control group ($U = 26.00$; $p = 0.004$), **as well as between the re-operated cleft patient group and the control group** ($U = 65.50$; $p = 0.029$). A smaller number of bFGF positive osteocytes were found in cleft bone tissue after the first and repeated surgery than in the control group.

The bFGF was observed in all cartilage tissue of patients in the control group. Its presence varied from few to moderate (+/++) to numerous (+++). In general, the number of bFGF positive chondrocytes in the cartilage tissue of the control group were moderate (++).

The bFGF was observed in all cartilage tissue of patients undergoing surgery for the first time. Its numbers varied from few to numerous (+/++) to abundant (++++). In general, bFGF positive chondrocytes were found to be numerous to abundant (+++/+++++) in the cartilage tissue of patients undergoing surgery for the first time (see Figure 2.10).

The bFGF was observed in the cartilage tissue of all reoperated cleft patients. The amount of bFGF positive chondrocytes varied from moderate (++) to abundant (++++). In general, numerous of bFGF positive chondrocytes were observed in the cartilage tissue of reoperated cleft patients (+++).

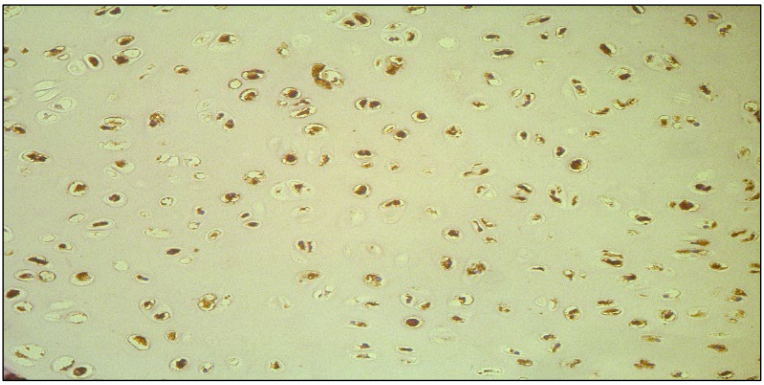

Figure 2.10 **Numerous to abundant (+++/++++) bFGF positive chondrocytes in cartilage tissue of a 6 years and 7 months old cleft first-time operated patient**
bFGF IMH, × 200

Statistics. In the case of bFGF, no statistically significant difference was observed between the cartilage tissue of cleft patients undergoing surgery for the first time and the cartilage tissue of cleft patients that had been re-operated. **A statistically significant difference was found both between the control group and the first-time operated cleft patient group** ($U = 37.50$; $p = 0.007$) **and between the reoperated cleft patient group and the control group** ($U = 35.50$; $p = 0.001$). This meant that the cartilage tissue of cleft-affected patients had significantly more bFGF positive chondrocytes in both the first-time and repeated surgeries than in the control group.

2.2.6 TGFβ1

The presence of TGFβ1 was observed in all bone tissue of patients in the control group. TGFβ1 positive osteocytes in the field of vision varied from few (+) to numerous (+++). In general, a few (+) TGFβ1 positive osteocytes were observed in the bone tissue of the control group.

The presence of TGFβ1 was observed in all bone tissue of cleft patients undergoing surgery for the first time. It ranged from rare (0/+) to numerous (+++) (see Figure 2.11). In general, in the bone tissue of patients undergoing cleft surgery for the first time, few (+) TGFβ1-positive osteocytes were observed.

TGFβ1 was observed in all bone tissue of reoperated cleft patients. Its results ranged from rare (0/+) to numerous (+++). In general, few to few to moderate (+ to +/++) TGFβ1 positive osteocytes were found in the bone tissue of re-operated cleft patients.

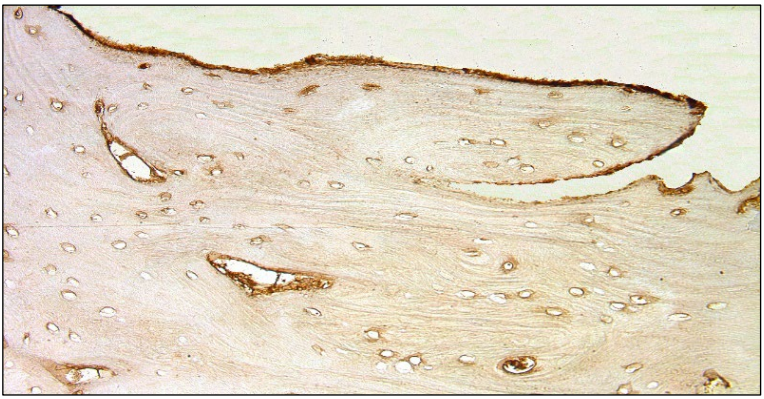

Figure 2.11 **Rare (0/+) TGFβ1 positive osteocytes in the bone tissue of 21 years and 1 month old in first-time operated cleft patient**
TGFβ1 IMH, × 200

Statistical data. No statistically significant difference was found between TGFβ1 positive osteocytes in the bone tissue of cleft patients first operated on and in bone tissue of re-operated cleft patients. Similarly, no statistically significant difference was observed between the control group and patients affected by cleft.

The presence of TGFβ1 was observed in all cartilage tissue of patients in the control group. Their findings ranged from few to moderate (+/++) to

numerous to abundant (+++/++++). In general, moderate to numerous TGFβ1 positive chondrocytes were observed in the control group's cartilage tissue (++/+++).

The presence of TGFβ1 was observed in all cartilage tissue of patients undergoing surgery for the first time. Its numbers varied from few (+) to abundant (++++) (see Figure 2.12). In general, numerous TGFβ1 positive chondrocytes (+++) were observed in patients undergoing surgery for the first time.

The presence of TGFβ1 was observed in all cartilage tissue of reoperated cleft patients. It ranged from moderate (++) to abundant (++++). In general, TGFβ1 positive chondrocytes were found to be numerous (+++) in the cartilage tissue of reoperated cleft patients.

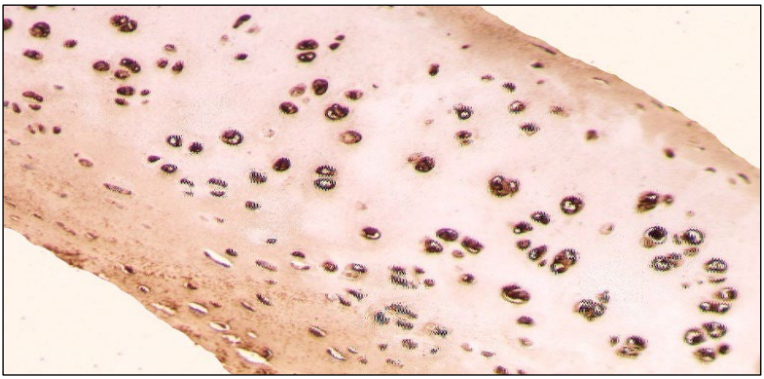

Figure 2.12 **Moderate to numerous (++/+++) TGFβ1-positive chondrocytes in cartilage tissue of 13 years and 1 month old first-time operated cleft patient**
TGFβ1 IMH, × 200

Statistical data. When evaluating the mean ranks of the relative numbers of TGFβ1 positive chondrocytes determined immunohistochemically, no statistically significant difference was observed between the study and control groups.

2.2.7 MMP-2

The presence of MMP-2 was observed in all bone tissue of patients in the control group. Overall, a few (+) MMP-2 positive osteocytes were observed in the bone tissue of the control group, however, the finding was variable. It ranged from rare (0/+) to moderate to numerous (++/+++) MMP-2 positive cells in the field of view.

The presence of MMP-2 was observed in all bone tissue of cleft patients undergoing surgery for the first time. It ranged from rare (0/+) MMP-2 positive osteocytes in the field of vision to moderate (++) MMP-2 positive osteocytes in the field of vision (see Figure 2.13). In general, MMP-2 positive osteocytes were observed rarely (0/+).

The presence of MMP-2 was not detected in all bone tissue of re-operated cleft patients. MMP-2 positive osteocytes were observed few to moderate (+/++). Their numbers ranged from no positive structures in the field of vision (0) to numerous positive structures in the field of vision (+++).

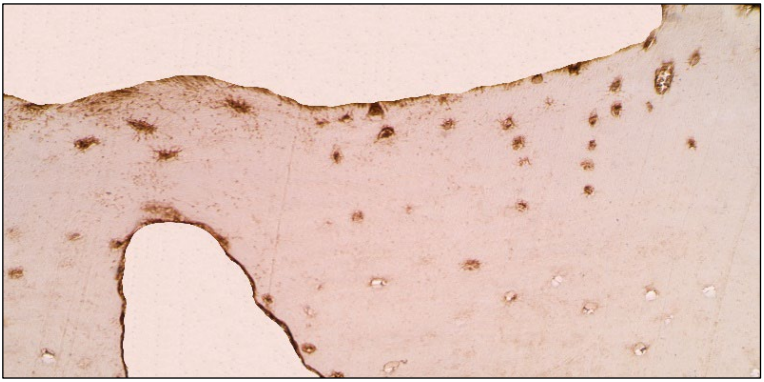

Figure 2.13 **Moderate (++) number of MMP-2 positive osteocytes in the bone tissue of 9 years and 11 months old cleft affected first-time operated patient**
MMP-2 IMH, × 200

Statistical data. **A statistically significant difference in MMP-2 positive osteocytes was found between first-time operated cleft patients' bone tissue and re-operated cleft patients' bone tissue**. This means that MMP-2 positive osteocytes are present in the vast majority in re-operated cleft patients bone tissue ($U = 95.50$; $p = 0.05$). No statistically significant difference was found between the control group and the two groups of cleft-affected patients with the Mann–Whitney U test.

The presence of MMP-2 was observed in all cartilage tissue of patients in the control group. In general, the control group's cartilage tissue MMP-2 positive chondrocytes were found to be moderate (++), however, the finding was variable. The number of positive chondrocytes varied from few to moderate (+/++) to numerous (+++).

The presence of MMP-2 was observed in all cartilage tissue of patients undergoing surgery for the first time. In general, in the cartilage tissue of patients undergoing surgery for the first time, MMP-2 positive chondrocytes were found to be numerous (+++) (see Figure 2.14). Their numbers varied from few to moderate (+/++) to abundant (++++).

The presence of MMP-2 was observed in all cartilage tissue of reoperated cleft patients. In general, numerous of MMP-2 positive chondrocytes (+++) were observed in the control group's cartilage tissue. Their numbers varied from few to moderate (+/++) to abundant (++++).

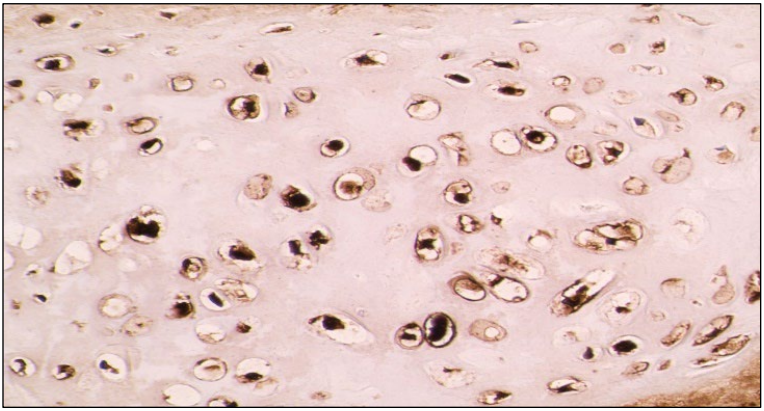

Figure 2.14 **Numerous (+++) MMP-2-positive chondrocytes in the cartilage tissue of 16 years and 6 months old cleft patient undergoing surgery for the first time**
MMP-2 IMH, × 250

Statistical data. The mean amount of chondrocytes containing MMP-2 in cartilage tissue **was statistically significantly higher in both first-time cleft-affected patients** ($U = 38.50$; $p = 0.008$) **and reoperated cleft patients** ($U = 37.00$; $p = 0.002$) compared to patients in the control group. No statistically significant difference in MMP-2 positive chondrocytes was observed between the cartilage tissue of first-time cleft patients and the cartilage tissue of reoperated cleft patients.

2.2.8 MMP-8

The presence of MMP-8 was detected in all bone tissue of patients in the control group. In general, MMP-8 positive osteocytes were observed in moderate (++) numbers.

MMP-8 positive osteocytes were found in all bone tissue of first-time operated cleft patients. They ranged from rare (0/+) to numerous (+++) (see Figure 2.15). Overall, MMP-8 positive osteocytes were detected few to few to moderate (+ to +/++).

MMP-8 positive osteocytes were not observed in all bone tissue of repeatedly operated cleft patients. Overall, MMP-8 positive osteocytes were found in few to few to moderate (+ to +/++). The number of MMP-8 positive osteocytes varied from no positive osteocytes in the field of vision (0) to numerous positive osteocytes in the field of vision (+++).

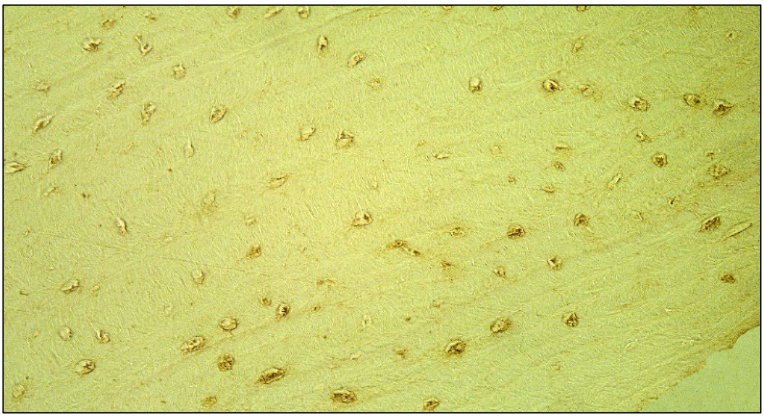

Figure 2.15 **Few (+) MMP-8 positive osteocytes in the bone tissue of 9 years and 5 months old first-time operated cleft patient**
MMP-8 IMH, × 250

Statistical data. No statistically significant difference in MMP-8 positive osteocytes was found between first-time operated cleft patients' bone tissue and re-operated cleft patients' bone tissue. Nor was there any statistically significant difference between the bone tissue of first-time operated cleft patients and the control group. However, **a statistically significant difference was found between the control group and the reoperated cleft patients group** ($U = 34.50$; $p = 0.025$), where there were fewer positive osteocytes in the MMP-8 group of reoperated cleft patients compared to the control group.

MMP-8 was detected in all cartilage tissue of patients in the control group. Its results ranged from moderate to numerous (++/+++) to abundant (++++). In general, in the control group's cartilage tissue, the MMP-8 positive chondrocytes were numerous to abundant (+++/++++).

MMP-8 positive chondrocytes were found in all cartilage tissue of first-time operated patients. They ranged from moderate (++) to abundant (++++) (see Figure 2.16). In general, numerous to abundant MMP-8 positive chondrocytes were observed in the cartilage tissue of patients undergoing surgery for the first time (+++/+++++).

MMP-8 positive chondrocytes were observed in all cartilage tissue of reoperated cleft patients. Their results ranged from moderate (++) to abundant (++++). In general, MMP-8 positive chondrocytes were found to be numerous to abundant (+++/++++) in the cartilage tissue of reoperated cleft patients.

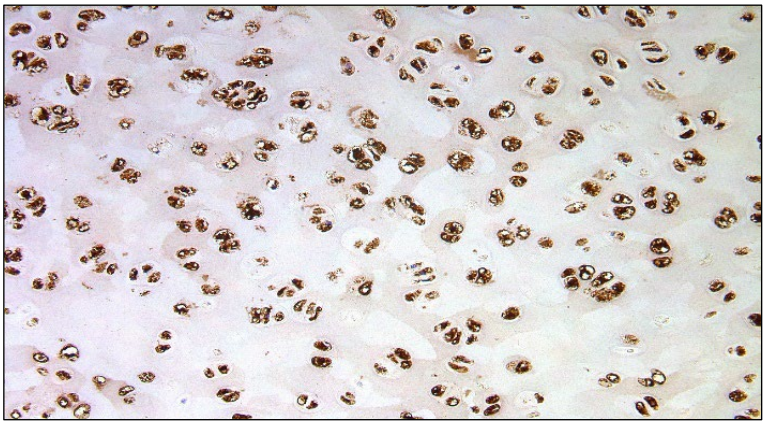

Figure 2.16 **Abundant (++++) MMP-8 positive chondrocytes in the cartilage tissue of 12 years and 8 months old first-time operated cleft patient**
MMP-8 IMH, × 400

Statistical data. It was found that the relative amount of average immunohistochemically determined MMP-8 positive chondrocytes in cartilage tissue did not differ statistically significantly between the study and control groups.

2.2.9 MMP-9

MMP-9 positive osteocytes were observed in all tissue samples of the control group. Their numbers ranged from rare (0/+) to moderate to numerous (++/+++). In general, in the bone tissue of the control group, MMP-9 positive osteocytes were observed in low to moderate numbers(+/++).

Osteocytes containing MMP-9 were observed in all bone tissue of cleft patients undergoing surgery for the first time. Their numbers ranged from rare (0/+) to moderate (++). In general, in the bone tissue of cleft patients undergoing surgery for the first time, few (+) MMP-9 positive osteocytes were observed (see Figure 2.17.).

The presence of MMP-9 was not detected in all bone tissue of repeatedly operated cleft patients. Overall, MMP-9 positive osteocytes in the bone tissue of re-operated cleft patients were few to few to moderate (+ to +/++), but their findings ranged from no positive structure (0) to numerous positive structures in the field of vision (+++).

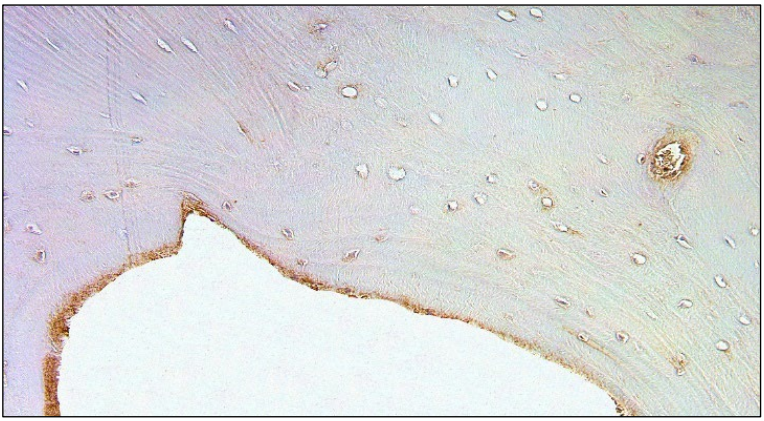

Figure 2.17 **Few (+) MMP-9 positive osteocytes the bone tissue of 8 years and 5 months old first-time operated cleft patient**
MMP-9 IMH, × 200

Statistical data. There was no statistically significant difference between MMP-9 positive osteocytes between first-time operated cleft patients' bone tissue and re-operated cleft patients' bone tissue. Nor was there a statistically significant difference found between the control group and the cleft patients.

The presence of MMP-9 was observed in all cartilage tissue of patients in the control group. The amount of positive chondrocytes in MMP-9 varied from few (+) to numerous (+++). In general, in the cartilage tissue of the control group, MMP-9 positive chondrocytes were observed in few to moderate numbers (+/++).

MMP-9 was observed in all cartilage tissue of patients undergoing surgery for the first time. The results of MMP-9 positive chondrocytes varied from moderate (++) to abundant (++++). In general, in the cartilage tissue of patients undergoing first-time surgery, MMP-9 positive chondrocytes were found to be numerous (+++) (see Figure 2.18).

The presence of MMP-9 was detected in all cartilage tissue of re-operated cleft patients. The number of positive chondrocytes varied from moderate (++) to numerous to abundant (+++/++++). In general, moderate to numerous

MMP-9 positive chondrocytes were observed in the cartilage tissue of re-operated cleft patients (++/+++).

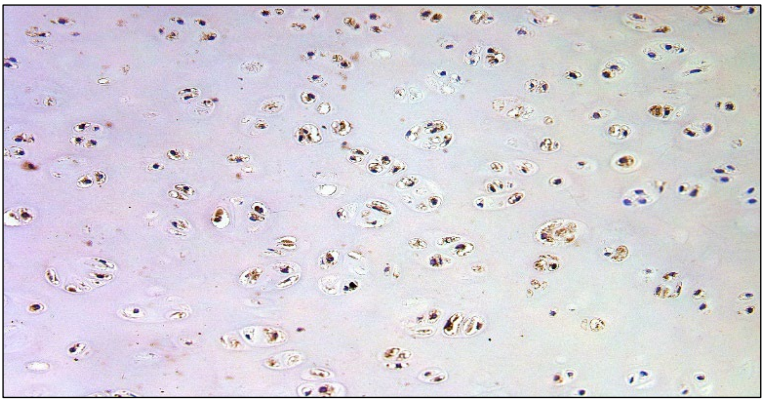

Figure 2.18 **Numerous (+++) MMP-9-positive chondrocytes in the cartilage tissue of 7 years and 7 months old first-time operated cleft patient**
MMP-9 IMH, × 400

Statistical data. In the case of the relative numbers of MMP-9 positive chondrocytes, no statistically significant difference was found between the cartilage tissue of first-time operated cleft patients and the cartilage tissue of re-operated cleft patients. **A statistically significant difference was found both between the control group and the first-time operated cleft patient group** ($U = 12.50$; $p < 0.001$) **and between the reoperated cleft patient group and the control group** ($U = 23.50$; $p < 0.001$). This meant that there were significantly more MMP-9-positive chondrocytes in the cartilage tissue tissue of cleft-affected patients after both the first and repeated surgeries than in the control group.

2.2.10 TIMP-2

The presence of TIMP-2 was observed in all bone tissue of patients in the control group. TIMP-2 positive osteocytes varied from rare (0/+) to moderate (++). In general, in the bone tissue of the control group, a few (+) TIMP-2 positive osteocytes were observed.

The presence of TIMP-2 was observed in all bone tissue of cleft patients undergoing surgery for the first time. It ranged from rare (0/+) to moderate (++) TIMP-2 positive osteocytes in the field of vision (see Figure 2.19). In general, TIMP-2 positive osteocytes were rarely observed in the bone tissue of cleft patients undergoing surgery for the first time (0/+).

TIMP-2 was observed in all bone tissue of reoperated cleft patients. Its results ranged from rare (0/+) to numerous (+++). In general, TIMP-2 positive osteocytes were rarely observed in bone tissue of reoperated cleft patients (0/+).

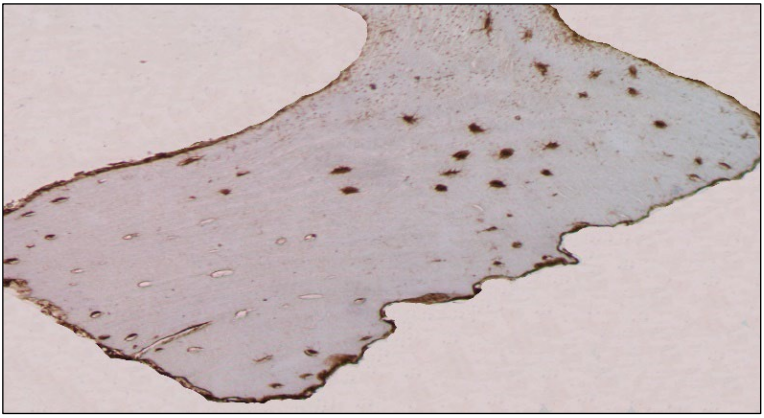

Figure 2.19 **Moderate (++) TIMP-2 positive osteocytes in the bone tissue of 6 years and 7 months old first-time operated cleft patient**
TIMP-2 IMH, × 200

Statistical data. In the case of this factor, no statistically significant difference was found between the bone tissue of cleft patients that had been

operated on for the first time and the bone tissue of the cleft patients that had been re-operated. No statistically significant difference was found between the control group and the two cleft-affected groups of patients with the Mann–Whitney U test.

TIMP-2 was observed in all cartilage tissue of patients in the control group. Its presence varied from few to moderate (+/++) to numerous to abundant (+++/++++). In general, in the cartilage tissue of the control group, TIMP-2 positive chondrocytes were found to be moderate to numerous (++/+++).

The presence of TIMP-2 was observed in all cartilage tissue of patients undergoing surgery for the first time. The amount of positive chondrocytes varied from few to moderate (+/++) to abundant (++++) (see Figure 2.20). In general, in the cartilage tissue of patients undergoing surgery for the first time, numerous TIMP-2 positive chondrocytes were observed (+++).

TIMP-2 was observed in all cartilage tissue of reoperated cleft patients. It ranged from few to moderate (+/++) to numerous to abundant (+++/++++). In general, in the cartilage tissue of reoperated cleft patients, TIMP-2 positive chondrocytes were observed moderate to numerous (++/+++).

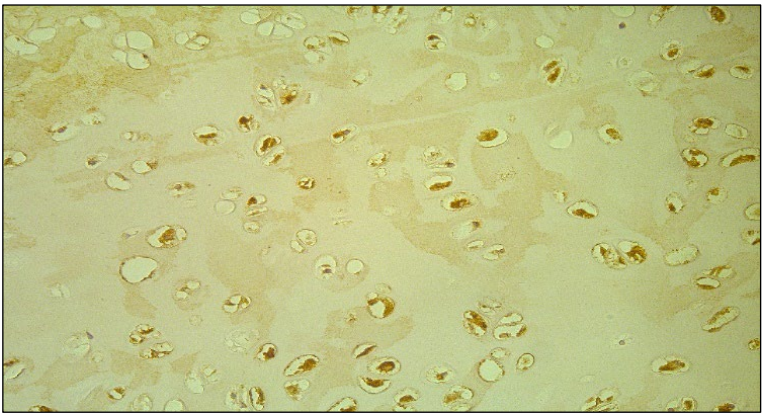

Figure 2.20 **Moderate (++) number of TIMP-2-positive chondrocytes in the cartilage tissue of 13 years and a 9 months old first-time operated cleft patient**
TIMP-2 IMH, × 400

Statistical data. In the case of this factor, no statistically significant difference was found between the cartilage tissue of cleft patients that had been operated on for the first time and the cartilage tissue of cleft patients that had been re-operated. No statistically significant difference was found between the control group and the two cleft-affected groups of patients with the Mann–Whitney U test.

2.2.11 IL-1α

The presence of IL-1α was not observed in all bone tissue of patients in the control group. In general, a few (+) IL-1α positive osteocytes were observed in the bone tissue of the control group.

Osteocytes containing IL-1α were observed in all bone tissue of cleft patients undergoing surgery for the first time. Their numbers varied from rare (0/+) to moderate (++). In general, IL-1α positive osteocytes were rarely observed in bone tissue of first-time operated cleft patients (0/+) (see Figure 2.21).

IL-1α was observed in all bone tissue of reoperated cleft patients. It varied from rare (0/+) to numerous (+++). Overall, there were few (+) IL-1α-positive osteocytes in the bone tissue of reoperated cleft patients.

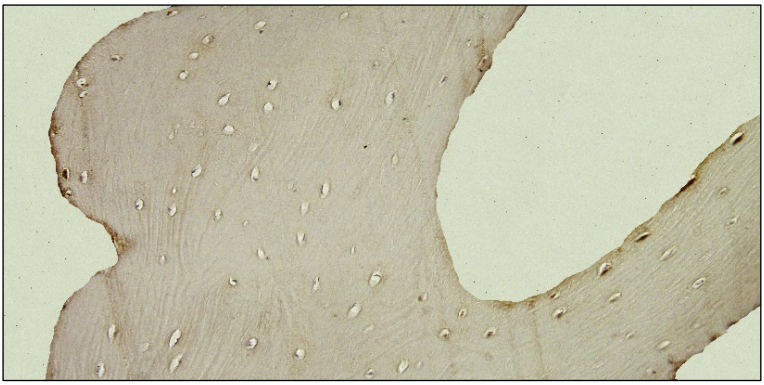

Figure 2.21 **Rare (0/+) IL-1α positive osteocytes in the bone tissue of a 13 years and 9 months old first-time operated cleft patient**
IL-1α IMH, × 200

Statistical data. When evaluating the mean ranks of the relative number of IL-1α determined immunohistochemically, no statistically significant difference between the study and control groups was observed.

IL-1α was detected in all cartilage tissue of patients in the control group. IL-1α positive chondrocytes varied from moderate (++) to abundant (++++). In general, the control group's cartilage tissue IL-1α positive chondrocytes were found to be moderate to numerous (++/+++).

The presence of IL-1α was detected in all cartilage tissue of first-time operated patients. Its results ranged from moderate (++) to a abundant (++++). In general, in the cartilage tissue of patients undergoing surgery for the first time, IL-1α positive chondrocytes were found to be numerous (+++) (see Figure 2.22).

IL-1α was detected in all cartilage tissue of reoperated cleft patients. Its numbers varied from moderate (++) to abundant (++++). In general, in the cartilage tissue of re-operated cleft patients, IL-1α positive chondrocytes have been observed to numerous (+++).

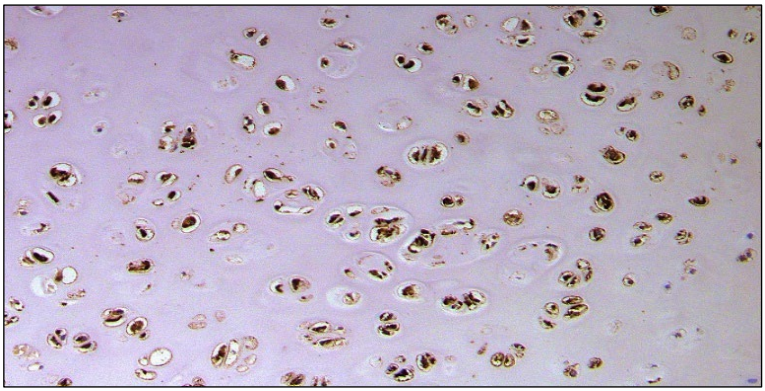

Figure 2.22 **Numerous (+++) IL-1α positive chondrocytes in the cartilage tissue of 18 years old first-time operated cleft patient**
IL-1α IMH, × 400

Statistical data. It was found that the relative amount of mean immunohistochemically determined IL-1α positive chondrocytes in cartilage tissue did not differ statistically significantly between the study and control groups.

2.2.12 IL-10

The presence of IL-10 was observed in all bone tissue of patients in the control group. In general, IL-10 positive osteocytes in bone tissue in the control group were observed in few to moderate amounts (+/++).

IL-10 positive osteocytes were observed in all bone tissue of cleft patients undergoing surgery for the first time. The number of osteocytes containing IL-10 varied from rare (0/+) to few to moderate (+/++) (see Figure 2.23).

In general, in the bone tissue of first-time operated cleft patients, a few (+) IL-10 positive osteocytes were observed.

The presence of IL-10 was observed in all bone tissue of repeatedly operated cleft patients. Its presence varied from rare (0/+) to numerous (+++). In general, there were few (+) IL-10 positive osteocytes in the bone tissue of re-operated cleft patients.

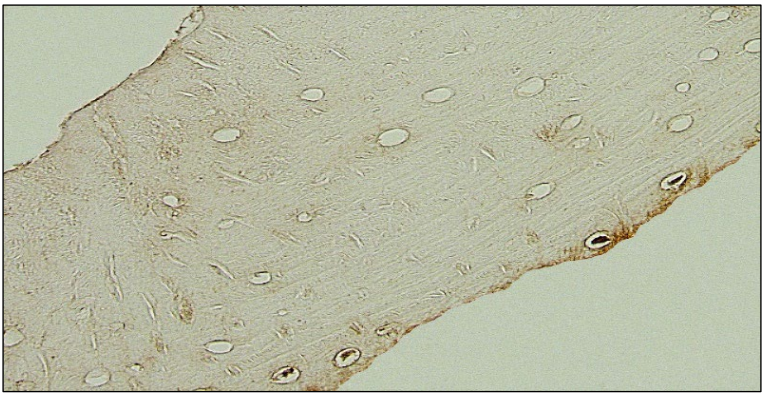

Figure 2.23 **Rare (0/+) IL-10 positive osteocytes in the bone tissue of 7 years and 5 months old first time operated cleft patient**
IL-10 IMH, × 250

Statistical data. **A statistically significant difference in the relative amount of IL-10 positive osteocytes in bone tissue was found between the control group and the first-time operated cleft patient group** ($U = 36.50$; $p = 0.022$), where there were fewer IL-10 positive osteocytes in the group of first-time operated cleft patients compared to the control group. There was no statistically significant difference between the other study and control groups.

The presence of IL-10 was observed in all cartilage tissue of patients in the control group. Its results ranged from few to moderate (+/++) to numerous (+++). In general, in the cartilage tissue of the control group, IL-10 positive chondrocytes were found to be in moderate number (++).

The presence of IL-10 was found in all cartilage tissue of patients undergoing surgery for the first time. Its numbers varied from moderate (++) to abundant (++++). In general, in the cartilage tissue of patients undergoing first-time surgery, IL-10 positive chondrocytes were found to be numerous (+++) (see Figure 2.24).

IL-10 was observed in all cartilage tissue of reoperated cleft patients. The results of the relative number of IL-10 positive chondrocytes varied from moderate (++) to abundant (++++). In general, in the cartilage tissue of re-operated cleft patients, IL-10 positive chondrocytes were found to be numerous (+++).

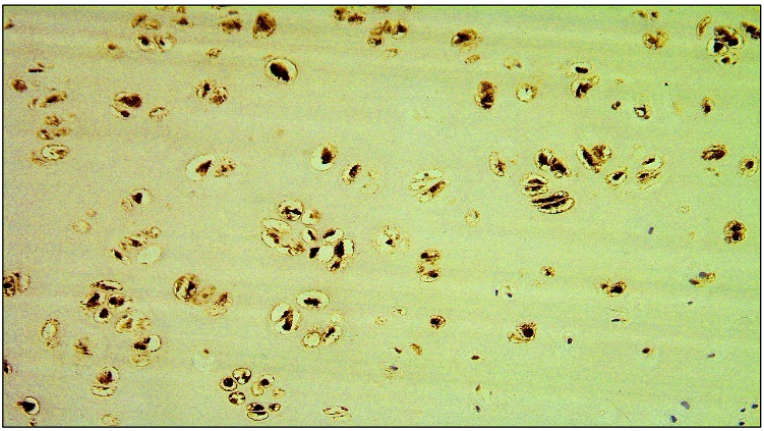

Figure 2.24 **Numerous (+++) IL-10 positive chondrocytes in the cartilage tissue of 5 years and 7 months old first-time operated cleft patient**
IL-10 IMH, × 400

Statistical data. In the case of IL-10, no statistically significant difference was found between the cartilage tissue of cleft patients undergoing first surgery and the cartilage tissue of re-operated cleft patients. **A statistically significant difference was found both between the control group and the first-time operated cleft patient group** ($U = 29.50$; $p = 0.002$) **and between**

the reoperated cleft patient group and the control group ($U = 58.00$; $p = 0.018$). This meant that there were significantly more IL-10 positive chondrocytes in the cartilage tissue of cleft-affected patients after both the first-time and repeated surgeries than in the control group.

2.2.13 HBD-2

HBD-2 was detected in all tissue samples of patients in the control group. Its presence varied from few (+) to numerous to abundant (+++/++++) HBD-2 positive osteocytes in the field of vision. In general, in the bone tissue of the control group, moderate number of HBD-2 positive osteocytes were observed (++).

The presence of HBD-2 was found in all bone tissue of cleft patients who had first been operated on. It ranged from rare (0/+) to moderate to numerous (++/+++) (see Figure 2.25). In general, in the bone tissue of first-time operated cleft patients, HBD-2-positive osteocytes were observed from rare to few (0/+ to +).

HBD-2 was detected in all bone tissue of reoperated cleft patients. Its numbers varied from rare (0/+) to numerous (+++). Overall, HBD-2 positive osteocytes were observed few (+) in the bone tissue of re-operated cleft patients.

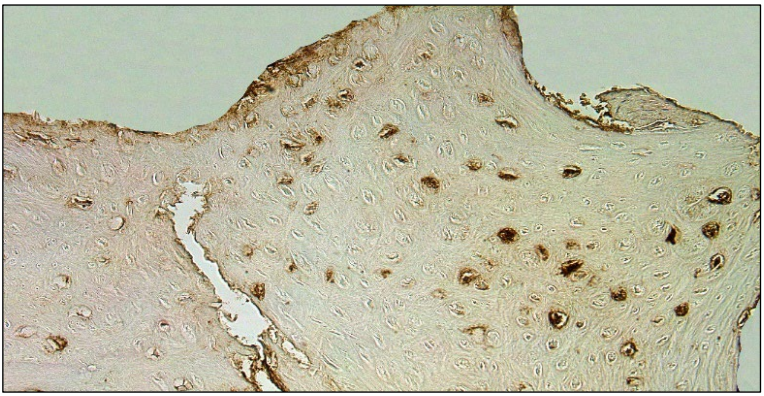

Figure 2.25 **Moderate (++) number of HBD-2 positive osteocytes in the bone tissue of a 13 years and 9 months old first-time operated cleft patient**
HBD-2 IMH, × 200

Statistical data. The mean amount of osteocytes containing HBD-2 in bone tissue **was statistically significantly lower in both first-time cleft-affected patients** ($U = 21.00$; $p = 0.002$) **and reoperated cleft patients** ($U = 53.00$; $p = 0.008$) compared to patients in the control group. A statistically significant difference in HBD-2 positive osteocytes between first-time operated bone tissue of cleft patients and re-operated bone tissue of cleft patients was not observed.

The presence of HBD-2 was observed in all cartilage tissue of patients in the control group. The amount of positive HBD-2 chondrocytes varied from few to moderate (+/++) to abundance (++++). In general, in the cartilage tissue of the control group, HBD-2 positive chondrocytes were in moderate number (++).

HBD-2 was observed in all cartilage tissue of patients undergoing surgery for the first time. Its relative numbers varied from moderate (++) to abundant (++++). In general, HBD-2 positive chondrocytes were numerous to abundant (+++/++++) in the cartilage tissue of first-time patients (see Figure 2.26).

The presence of HBD-2 was observed in all cartilage tissue of reoperated cleft patients. Its results ranged from few to moderate (+/++) to abundant (++++). In general, moderate to numerous HBD-2 positive chondrocytes were found in the cartilage tissue of re-operated cleft patients (++/+++).

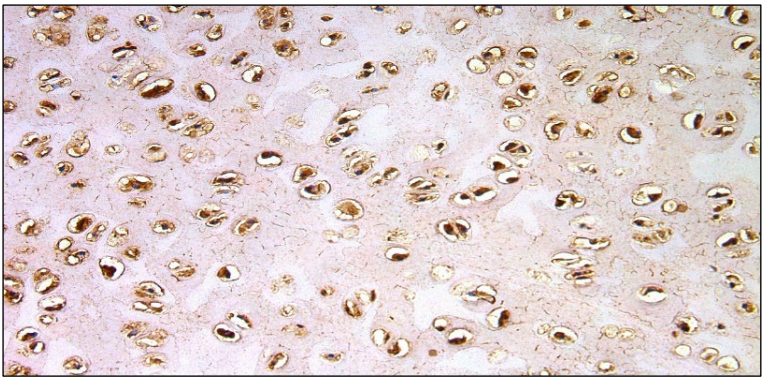

Figure 2.26 **Moderate to numerous (+++/++++) HBD-2 positive chondrocytes of 11 years and 9 months old first-time operated cleft patient**
HBD-2 IMH, × 250

Statistical data. When evaluating the mean ranks of the relative amount of HBD-2 positive chondrocytes determined immunohistochemically, no statistically significant difference was observed between the study and control groups.

2.2.14 HBD-3

The presence of HBD-3 was observed in all bone tissue of patients in the control group. HBD-3 positive osteocytes in the field of vision varied from few (+) to numerous to abundant (+++/++++). In general, HBD-3 positive osteocytes in bone tissue in the control group were observed in few to moderate amounts (+/++).

HBD-3 positive osteocytes were observed in all bone tissue of cleft patients undergoing surgery for the first time. The amount of osteocytes containing HBD-3 varied from rare (0/+) to moderate (++) (see Figure 2.27). In general, HBD-3 positive osteocytes were observed from rare to few (0/+ to +) in bone tissue of cleft patients with the first-time surgery.

HBD-3 was not detected in all bone tissue of reoperated cleft patients. Its results ranged from no positive osteocytes (0) to moderate (++) number of HBD-3 positive osteocytes in the field of vision. Overall, HBD-3 positive osteocytes were few (+) in the bone tissue of re-operated cleft patients.

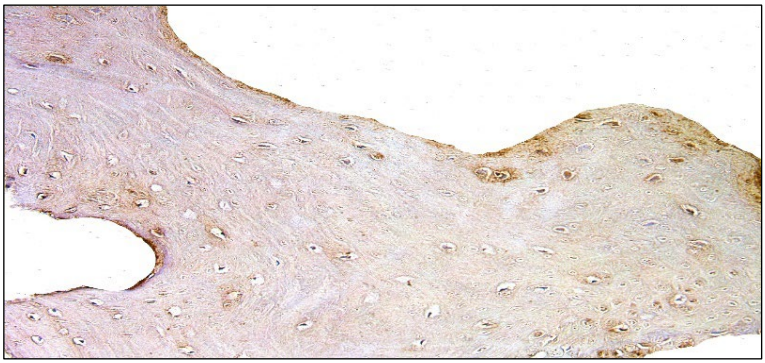

Figure 2.27 **Few (+) HBD-3 positive osteocytes in the bone tissue of 14 years and 1 month old first-time operated cleft patient**
HBD-3 IMH, × 200

Statistical data. In the case of this factor, no statistically significant difference in the amount of HBD-3 positive osteocytes was found between the bone tissue of cleft patients who had undergone first-time surgery and the bone tissue of cleft patients who had been re-operated. However, **a statistically significant difference was found both between the control group and the first-time operated cleft patient group** ($U = 22.00$; $p = 0.002$) **and between the cleft patient group and the control group** ($U = 41.00$;

p = 0.002). This meant that there were significantly fewer HBD-3 positive osteocytes in the bone tissue of patients affected by cleft after both the first and repeated surgeries than in the control group.

HBD-3 was detected in all cartilage tissue of patients in the control group. Its number varied from few (+) to numerous (+++). In general, HBD-3 positive chondrocytes were observed in moderate number (++) in the cartilage tissue of the control group.

The presence of HBD-3 was observed in all cartilage tissue of patients undergoing surgery for the first time. It ranged from moderate (++) to abundant (++++). In general, moderate to numerous HBD-3 positive chondrocytes were observed in the cartilage tissue of patients undergoing surgery for the first time (++/+++) (see Figure 2.28).

The presence of HBD-3 was found in all cartilage tissue of reoperated cleft patients. It ranged from moderate (++) to abundant (++++). In general, numerous HBD-3 positive chondrocytes were observed in the cartilage tissue of re-operated cleft patients (+++).

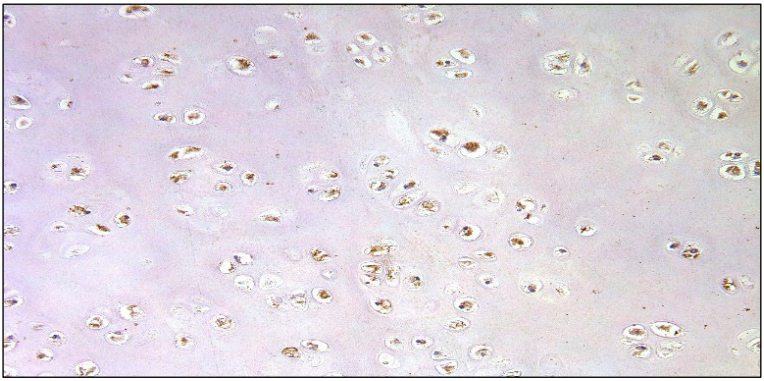

Figure 2.28 **Moderate to numerous (++/+++) HBD-3-positive chondrocytes in the cartilage tissue of 8 years and 3 months old first-time operated cleft patient**
HBD-3 IMH, × 400

Statistical data. The relative amount of HBD-3 positive chondrocytes showed no statistically significant difference between first-time and re-operated cleft patients' cartilage tissue. **A statistically significant difference was found both between the control group and the first-time operated cleft patient group** ($U = 45.50$; $p = 0.019$) **and between the re-operated cleft patient group and the control group** ($U = 63.00$; $p = 0.030$). This meant that there were significantly more HBD-3 positive chondrocytes in the cartilage tissue of cleft-affected patients after both the first and repeated surgeries than in the control group.

2.2.15 Runx2

Runx2 was not present in all bone tissue in the control group. In general, Runx2 positive osteocytes were not observed in bone tissue in the control group (0).

Osteocytes containing Runx2 were not observed in all bone tissue of cleft patients undergoing surgery for the first time. In general, Runx2 positive osteocytes were rarely observed in the bone tissue of cleft patients with surgery for the first time (0/+) (see Figure 2.29).

Runx2 was not detected in all bone tissue of reoperated cleft patients. Its numbers varied from no visible structures in the field of vision (0) to few to moderate (+/++) number of positive structures in the field of view. In general, Runx2 positive osteocytes were rare (0/+) in bone tissue in reoperated cleft patients.

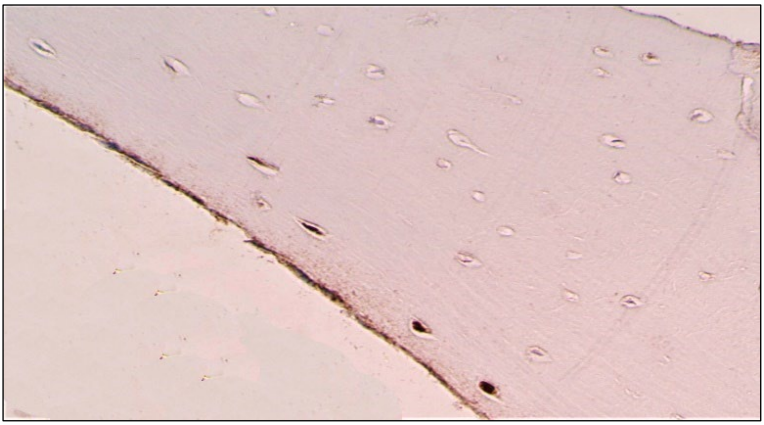

Figure 2.29 **Rare (0/+) Runx2 positive osteocytes in the bone tissue of 7 years and 5 months old a cleft patient who has undergone surgery for the first time**
Runx2 IMH, × 250

Statistical data. It was found that the relative number of average immunohistochemically determined Runx2 positive osteocytes in bone tissue did not differ statistically significantly between the study and control groups.

Runx2 was observed in all cartilage tissue of patients in the control group. Its numbers varied from few (+) to numerous (+++). In general, in the control group's cartilage tissue, moderate (++) number of Runx2 positive chondrocytes were observed.

The presence of Runx2 was detected in all cartilage tissue of patients undergoing surgery for the first time. Its results ranged from rare (0/+) to numerous (+++). In general, Runx2 positive chondrocytes were detected on moderate number (++) in the cartilage tissue of patients undergoing surgery for the first time (see Figure 2.30).

The presence of Runx2 was detected in all cartilage tissue of reoperated cleft patients. Its results ranged from rare (0/+) to numerous (+++). In general, in

the cartilage tissue of re-operated cleft patients, Runx2-positive chondrocytes were found to be few (+).

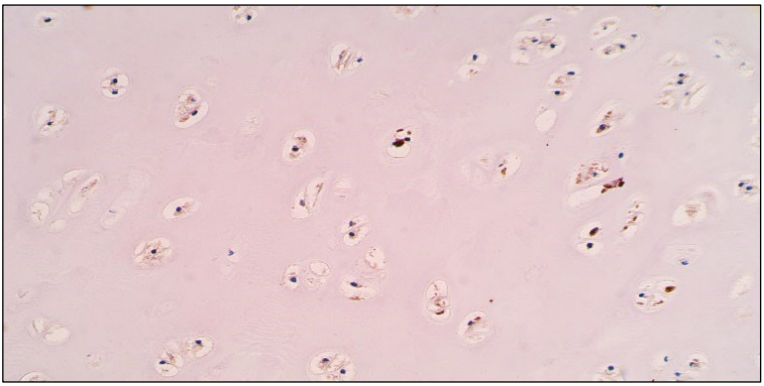

Figure 2.30 **Moderate number (++) of Runx2 positive chondrocytes in the cartilage tissue of 5 years and 7 months old first-time operated cleft patient**
Runx2 IMH, × 400

Statistical data. It was found that the relative number of average immunohistochemically determined Runx2-positive chondrocytes in cartilage tissue did not differ statistically significantly between the study and control groups.

2.2.16 Wnt3a

Wnt3a was not present in all patients in the control group. In general, rare (0/+) Wnt3a positive osteocytes were observed in bone tissue in the control group.

Wnt3a was present in all bone tissue of cleft patients undergoing surgery for the first time. It ranged from rare (0/+) to moderate (++) (see Figure 2.31). In general, in the bone tissue of patients with first-time cleft surgery, a few (+) Wnt3a positive osteocytes were observed.

Wnt3a was not present in all bone tissue of reoperated cleft patients. It ranged from non-visible structures in the field of view (0) to numerous (+++) positive structures in the field of view. In general, a few (+) Wnt3a positive osteocytes were observed in the bone tissue of reoperated cleft patients.

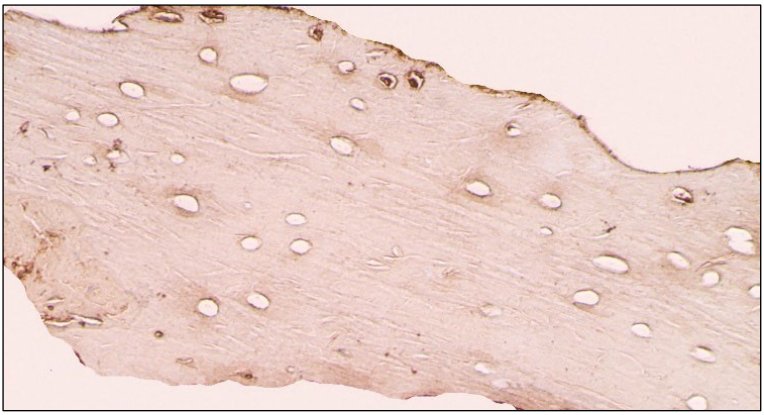

Figure 2.31 **Rare (0/+) Wnt3a positive osteocytes in the bone tissue of an 8 year old cleft patient who has undergone surgery for the first time**
Wnt3a IMH, × 200

Statistical data. The relative amount of average immunohistochemically determined Wnt3a positive osteocytes in bone tissue did not differ statistically significantly between the study and control groups.

Wnt3a was present in all cartilage tissue of patients in the control group. Wnt3a's number varied from moderate (++) to numerous to abundant (+++/++++). In general, in the cartilage tissue of the control group, Wnt3a positive chondrocytes were moderate to numerous (++/+++).

Wnt3a was observed in all cartilage tissue of patients undergoing surgery for the first time. Its relative numbers varied from few (+) to abundant (++++). In general, numerous Wnt3a positive chondrocytes were observed (+++) in

the cartilage tissue of patients undergoing surgery for the first time (see Figure 2.32).

Wnt3a was observed in all cartilage tissue of reoperated cleft patients. Its numbers varied from moderate (++) to abundant (++++). In general, numerous Wnt3a positive chondrocytes were observed in the cartilage tissue of reoperated cleft patients (+++).

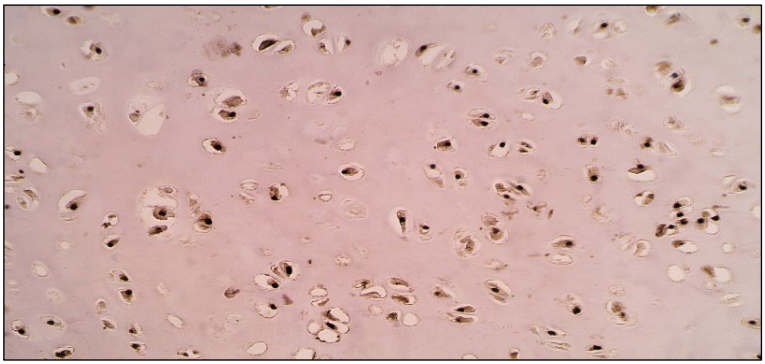

Figure 2.32 **Numerous (+++) Wnt3a-positive chondrocytes in the cartilage tissue of 6 years and 7 months first-time operated cleft patient**
Wnt3a IMH, × 400

Statistical data. In the case of this factor, no statistically significant difference was found between the cartilage tissue of first-time and reoperated cleft patients. A statistically significant difference between the control group and the two groups of cleft-affected patients with the Mann–Whitney U test was also not found.

2.2.17 Apoptose

Apoptotic cells were observed in all bone tissue of patients in the control group. Apoptosis positive osteocytes in the field of vision varied from few (+) to

numerous (+++). In general, moderate (++) number of apoptotic osteocytes were observed on average in bone tissue in the control group.

Apoptotic osteocytes were observed in all bone tissue of cleft patients undergoing surgery for the first time. The amount of apoptotic osteocytes in the field of vision varied from rare (0/+) to numerous (+++) (see Figure 2.33). In general, in the bone tissue of first-time operated cleft patients, apoptotic osteocytes were observed few to few to moderate (+ to +/++).

Apoptotic cells were found in all bone tissue of reoperated cleft patients. Their presence varied from rare (0/+) to numerous (+++). In general, apoptosis positive osteocytes in the bone tissue of reoperated cleft patients were found to be few to moderate (+/++).

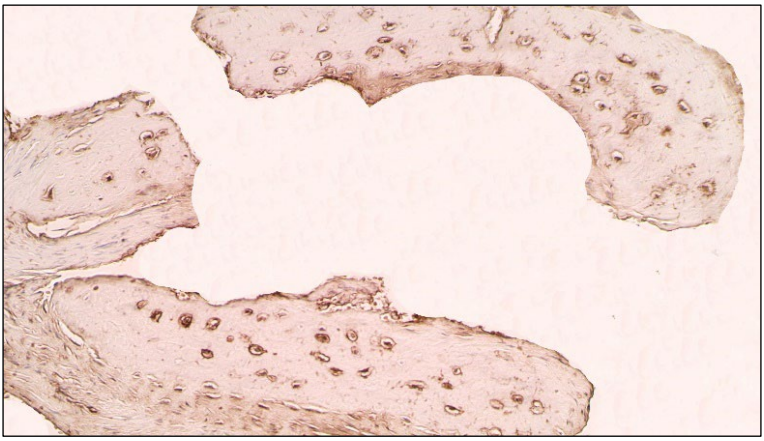

Figure 2.33 **Numerous (+++) apoptosis positive osteocytes in the bone tissue of 8 years and 1 month old first-time operated cleft patient**
TUNEL, × 200

Statistical data. In the case of apoptosis, no statistically significant difference was found between the bone tissue of the cleft patients undergoing surgery for the first time and the bone tissue of the cleft patients that had been

re-operated. **A statistically significant difference was found both between the control group and the first-time operated cleft patient group** ($U = 33.00$; $p = 0.013$) **and between the reoperated cleft patient group and the control group** ($U = 37.50$; $p = 0.001$). This meant that the bone tissue of patients affected by cleft had significantly fewer apoptosis positive osteocytes after both the first and repeated surgeries than in the control group.

Apoptotic cells were observed in all cartilage tissue in the control group. The results of the relative number of apoptosis positive chondrocytes varied from moderate (++) to numerous to abundant (+++/++++). In general, apoptosis positive chondrocytes in the cartilage tissue of the control group were observed moderate to numerous (++/+++).

Apoptotic cells were observed in all cartilage tissue of patients undergoing surgery for the first time. The amount of apoptosis positive chondrocytes varied from few to moderate (+/++) to abundant (++++). In general, apoptosis positive chondrocytes in the cartilage tissue of first-time operated patients were found to be moderate to numerous (++/+++) (see Figure 2.34).

Apoptotic cells were observed in all cartilage tissue of reoperated cleft patients. Their results ranged from an moderate (++) to a numerous (+++). In general, moderate (++) number of apoptosis positive chondrocytes were observed in the cartilage tissue of reoperated cleft patients.

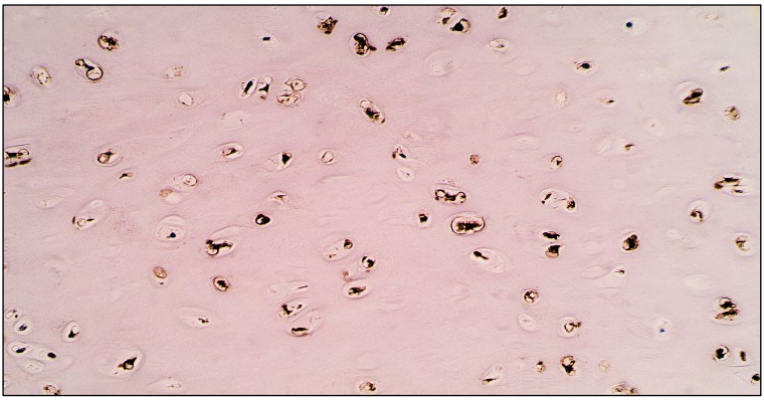

Figure 2.34 **Moderate to numerous (++/+++) apoptosis positive chondrocytes in the cartilage tissue of 7 year old first-time operated cleft patient**
TUNEL, × 400

Statistical data. **A statistically significant difference was found in apotosis positive chondrocytes between first-time operated cleft patients' cartilage tissue and re-operated cleft patients' cartilage tissue**. This means that apoptosis positive chondrocytes are present in the overwhelming majority of first-time cleft patients in cartilage tissue ($U = 107.50$; $p = 0.021$). Similarly**, a statistically significant increase in apoptosis positive chondrocytes was observed in the control group compared to the cartilage tissue of re-operated cleft-affected patients** ($U = 63.00$; $p = 0.016$). No statistically significant difference was found between the control group and the group of patients affected by the cleft that had been operated for the first time with the Mann–Whitney U test.

2.3 Data from the evaluation of immunohistochemically determined marker correlations in bone tissue for the first time and reoperated cleft patients

There were 16 strong and 38 moderately strong, positive correlations in the bone tissue of first-time operated cleft patients. Of the statistically significant, strong, positive correlations, they were most commonly found with TIMP-2, BMP-2/4, IL-10, and Wnt3a. No statistically significant, strong positive correlations were found for MMP-8, MMP-9, bFGF, IL-1α, HBD-3, and apoptosis. Of the statistically significant, moderately strong, positive correlations, they were most commonly found with bFGF, HBD-2, IL-1α, and OPN. The smallest amount of statistically significant, moderately strong, positive correlations was found with MMP-9, Runx2, and Wnt3a.

In the bone tissue of reoperated cleft patients, 52 strong and 70 moderately strong, positive correlations were found. Of the statistically significant, strong, positive correlations, they were most often found with bFGF, HBD-2, and IL-10. The fewest correlations were found with TIMP-2 and MMP-2. No statistically significant, strong positive correlations were found in Runx2. Of the statistically significant, moderately strong, positive correlations, they were most commonly found with MMP-8, IL-1α, and Runx2. The least statistically significant, moderately strong, positive correlation was found with MMP-9 and OPG.

2.4 Data from the evaluation of immunohistochemically determined marker correlations in cartilage tissue for the first time and reoperated cleft patients

For the first time, 42 strong and 24 moderately strong, positive correlations were found in the cartilage tissue of operated cleft patients. From statistically significant, strong, positive correlations, they were most commonly found with BMP-2/4, bFGF, and IL-1α. No statistically significant, strong positive correlations were found for MMP-2, MMP-9, Runx2, and apoptosis.

Of the statistically significant, moderately strong, positive correlations, they were most commonly found with HBD-3 and OPN. The smallest amount of statistically significant, moderately strong, positive correlations was found with bFGF, MMP-2, MMP-8, BMP-2/4, HBD-2, and apoptosis. Statistically significant, strong positive correlations of Runx2 were not found in the cartilage tissue of the first operated cleft patients.

In the cartilage tissue of re-operated cleft patients, 7 strong and 42 moderately strong, positive correlations were found. Of the statistically significant, strong, positive correlations, they were most often found with TGFβ1 and IL-10. The fewest correlations were found with BMP-2/4, OPG, and HBD-3. Of the statistically significant, moderately strong, positive correlations, they were most commonly found with MMP-2 and IL-1α. The least statistically significant, moderately strong, positive correlation was found with TIMP-2, Runx2, and apoptosis.

3 Discussion

Studies are being conducted in many parts of the world on the population of patients affected by facial clefts, however, there are not many studies that look directly at cleft supporting tissue (bone and cartilage tissue). What makes this study unique is that the bone and cartilage tissue and their changes were first viewed after both the first-time and repeated surgeries. Similarly, there is not much comprehensive data in the world on immunohistochemical data of various tissue factors and apoptosis distribution in the supporting tissues of cleft-affected patients. Our study evaluated the relative number of OC, OPN, OPG, BMP-2/4, bFGF, TGFβ1, MMP-2, MMP-8, MMP-9, TIMP-2, IL-1α, IL-10, HBD-2, HBD-3, Runx2, Wnt3a, and apoptosis positive osteocytes and chondrocytes in cleft-affected bone and cartilage tissue after both their first-time and repeated surgery.

In general, the morphological finding of bone and cartilage tissue in cleft-affected patients was in line with the generally accepted norm. However, osteons of various sizes were observed in the bone tissue of cleft-affected patients, their Haversion channels were filled with connective tissue, which could indicate changes in the vascularisation and innervation of the bone. The bone plates were of heterogeneous colour and irregular size, which could indicate a change in bone strength. In the cartilage tissue of patients affected by cleft, all areas of cartilage were observed, and they corresponded to the generally accepted morphological norm.

OC is a protein that is mainly expressed by osteoblasts. It is a specific, non-collagen ECM protein controlled by the transcription factor Runx2/Cbfa1 (Oury and Oury, 2018). OC is located in the densely mineralised area of the cortical bone (Weinreb et al., 1990). It regulates bone remodeling by modulating the activity of osteoblasts and osteoclasts, and is also a factor in bone mineralisation, which plays a role in calcium metabolism (Zhang, 2023).

The results obtained in our study groups indicated the presence of OC in all bone and cartilage tissues. Numerous OC positive osteocytes and chondrocytes were found in the bone and cartilage tissue control group. However, statistically significant differences were observed between first-time surgery and re-operated cleft patients' bone tissue compared to the control group, where a lower number of OC positive osteocytes was found in cleft bone tissue. Since OC is considered one of the factors influencing bone growth and regeneration, its numbers could reflect the degree of osteoblast activity and the nature of bone metabolism (Cundy et al., 2014), where its absence can lead to a significant decrease in bone strength and the correct positioning of collagen fibers in the bone (Yildirim et al., 2023).

Earlier studies on OC deficient mice showed that loss of OC function inhibits bone resorption and suppresses the genesis of osteoclasts. As a result of this process, bone mass increased, with no effect on the mineralisation process (Ducy et al., 1996; Boskey et al., 1998). However, further research has shown that OC has no effect on bone formation, or its mass, but rather plays a role in the position of hydroxyapatite crystals alongside collagen fibers (Moriishi et al., 2023). Disturbances in the arrangement of these crystals in OC deficient mice lead to changes in the mineral composition of the bone that affect the integration of the bone structure, making it an important factor in the process of bone mineralisation (Diegel et al., 2020; Xu et al., 2023). In addition, the loss of OC function does not affect the orientation of collagen. However, in OC deficiency mice, bone strength was reduced (Manolagas, 2020). The results of our study could be explained by the reduced mineralisation potential of cleft-affected bone tissue, which is likely to indicate a loss of the mechanical properties of the bone and, consequently, a more labile tissue change during surgical intervention (Monir, 2010).

OC can also be found in chondrocytes. OC containing chondrocytes, along with the cartilage ECM, participate in this process (Farnum and Wilsman, 1987). OC expression in chondrocytes indicates the differentiation of chondrocytes in a hypertrophic phenotype, which is a physiological and necessary process after an injury, with subsequent cartilage mineralisation (Pullig et al., 2000).

In the cleft groups of cartilage tissue we studied, as in the control group, numerous OC positive chondrocytes were found with no statistically significant difference between the groups. This could be explained by the better mechanical strength capacity of cartilage tissue compared to bone tissue after both first and repeated surgical interventions, since this ability is not significantly different from healthy tissue.

OPN is a phosphoprotein that can be found in the ECM of mineralised tissues. OPN binds to calcium and provides initial mineralisation (Botham and Murray, 2018). It is secreted by many types of cells, such as osteoclasts, chondrocytes, macrophages, lymphocytes, epithelial cells and vascular smooth muscle cells (Denhardt et al., 1998). In addition, OPN is associated with bone remodeling and mineral density, which affects bone formation (Cho et al., 2013).

In our study, a difference in the number of OPN positive osteocytes was found between the control group and the two groups of patients affected by the clefts. The relative amount of positive OPN osteocytes in cleft tissues was low, but it was even lower in the control group. OPN positive cells were also statistically more significant in both groups of cleft-affected cartilage tissue compared to the control group. However, compared to bone tissue, the amount of OPN positive structures in cartilage tissue was higher. Higher OPN levels have been observed in tissues with low bone density, which may be associated with fracture formation (Fodor et al., 2013; Vancea et al., 2021), progressive joint damage, (Martinez-Calleja et al., 2014), cartilage degeneration and bone

remodeling (Lin et al., 2022). The differences found in our study data between cleft-affected bone and cartilage tissues and tissues of control groups could indicate increased bone and cartilage homeostasis on the background of a reduced mineralisation process. This could indicate a possible reaction of cleft-affected bone and cartilage tissue compensatory to the effects of trauma.

A 2021. study showed that inhibiting OPN and inflammatory cytokines delays the further development of OA (Li et al., 2021). It was also observed that elevated levels of OPN and MMP-9 in OA are stimulated by cytokines IL-1 and IL-6 (Slovacek et al., 2020). These data also coincide with the results of our study, where moderately strong, positive correlations were found in cleft-affected bone tissue groups, both in the bone tissue of first-operated cleft patients between OPN and MMP-9 and between OPN and IL-1α, and in the bone tissue of reoperated cleft patients between OPN and MMP-9 and OPN and IL1α. In cleft-affected cartilage tissue, OPN showed a moderately strong, positive correlation with IL-1α after the first surgery, but after repeated surgery with IL-1α and MMP-9. These data may indicate increased bone and cartilage remodeling abilities in patients with cleft.

OPG is the main modulator of osteoclast genesis, also known as an inhibitor of osteoclast terminal differentiation and activation (Hsu et al., 2006). OPG is expressed by osteoblasts and osteocytes. It interacts with the nuclear factor κB ligand (RANKL) receptor activator, inhibiting the binding of RANKL to RANK and thus promoting osteoclastogenesis and inhibiting bone resorption (Kwan et al., 2008; Nakashima et al., 2011).

When analyzing the OPG finding, we did not observe statistically significant differences between the study groups. However, compared to bone tissue, the relative number of OPG was higher in cartilage tissue. The presence of a greater number of OPG could be a cartilage tissue protection process, as increased OPG levels reduce osteoclast activity, which could lead to less

cartilage damage. In cleft affected cartilage tissue, strong, positive correlations were observed with bFGF and TGFβ1, which could indicate an increased process of cartilage tissue proliferation. There is evidence that endogenous OPG protects against cartilage degradation in OA (Shimizu et al., 2007). However, contrary to this, Takegami et al. found that OPG can also participate in the progression of cartilage degeneration, which is observed in inflamed introvertebral discs (Takegami et al., 2017). In contrast, 2019. study showed that reduction in OPG/RANKL in cartilage leads to chondrocyte apoptosis and loss of ECM through the MMP activation mechanism (Kovacs et al., 2019).

OPG protects the bone from excessive resorption by binding to RANKL and preventing it from attaching to RANK. Thus, the relative concentration of RANKL and OPG in bones is an important determinant of bone mass and strength (Boyce et al., 2008). The low relative number of OPG in bone tissue in our study, as well as strong, positive correlations with apoptosis, could indicate increased bone resorption and a decrease in bone mass in cleft-affected bone tissue, which could potentially be a poor prognosis for bone healing and regeneration after surgical intervention.

Throughout life, bone remodeling is a continuous process. This is ensured by the balance between osteoblasts, which are important in the process of bone formation, and osteoclasts, which play a role in the process of bone resorption (Huntley et al., 2019). **BMP-2/4** is a growth factor belonging to class TGFβ. It is a powerful stimulator of osteogenesis and chondrogenesis, with effects on the formation and activation of osteoblasts (Capulli et al., 2014; Wu et al; 2016).

Our results revealed the expression of BMP-2/4 in all bone and cartilage tissue, which justifies its vital role in maintaining bone and cartilage homeostasis. BMP-2/4 positive osteocytes were statistically significantly less present in the bone tissue of patients with surgery for the first time than in the control group. This could indicate that the cleft defect influences BMP-2/4 expression or

activity in bone tissue. These data could also indicate that bone regeneration and successful wound healing are impaired in these patients. Similar data exist in other studies where the lack of BMP led to osteopenia and decreased bone formation (Devlin et al., 2003; Medici et al., 2006). However, although BMP-2/4 positive osteocytes were observed more in the bone tissue of reoperated patients than in the bone tissue of first-time operated patients, no statistically significant difference between the groups was found. This could indicate that the surgical intervention itself does not affect the level of BMP-2/4 as significantly as it is affected by a cleft defect. Other studies have shown that BMP levels increase in bone resorption, contributing to the genesis of osteoblasts and the formation of a new bone (Charles et al., 2014), as well as, at the site of a bone fracture, in the event of an injury (Spector et al., 2001). These results coincide with the data obtained in our study that the level of BMP-2/4 in the bone tissue affected by the cleft after their repeated surgery, increases, possibly under the influence of repeated injury.

A higher number of BMP-2/4 positive chondrocytes was observed in cleft patient groups than in the control group, but a statistically significant difference was only observed between reoperated cleft patients and the control group. BMP plays a well-known role in chondrogenesis, contributing to the proliferation, differentiation, and maturation of chondrocytes (Chen et al., 1997). The increased expression of BMP-2/4 in the cartilage tissue of cleft patients suggests its active role in the recovery process after surgery, which could act as a compensatory mechanism after a surgical injury. This coincides with data from a 2006. study when, under fractured conditions, BMP stimulated cartilage regeneration (Kuo et al., 2006). The increased BMP-2/4 amount of cartilage tissue compared to bone tissue could indicate a higher potential for cartilage regeneration and healing than bone tissue.

BMP-2/4 showed positive correlations with TIMP-2, HBD, IL-10, and Wnt3a in both bone and cartilage tissue groups, which could indicate preserved bone and cartilage regeneration and plasticity, as well as wound-healing abilities in cleft tissues.

In conclusion, our study provides valuable insights into the amount of BMP-2/4 found in bone and cartilage tissue in patients with cleft palate and control groups. While the exact mechanisms that determine this finding have yet to be clarified, our findings emphasise the importance of BMP-2/4 in regeneration processes after palate remodeling. At the same time, these data highlight the potential therapeutic value of BMP-2/4, which could be used to improve tissue regeneration and surgical treatment outcomes in patients with cleft palate.

bFGF plays a vital role in the development and regeneration of bone and cartilage tissue. It participates in many biological processes, including embryonic development, cell growth, morphogenicity, wound healing and tissue renewal, stimulating the formation of new blood vessels (Presta et al., 2018; Zhang X et al., 2018). bFGF stimulates the differentiation and proliferation of osteogenic cells that play a role in the formation and mineralisation of bone matrix (Du et al., 2017). bFGF is one of the most widely studied factors in relation to wound healing (Guo et al., 2006; Yang et al., 2004). Through the enhancement of other growth factors such as TGFβ and BMP, bFGF also stimulates chondrocyte proliferation and ECM synthesis in cartilage tissue, including accelerating cartilage repair (Li et al., 2013). Thus, changes in bFGF levels could potentially affect the homeostasis and integrity of bones and cartilage.

In our study, the presence of bFGF was found in all study groups. Data from study groups suggest that the relative number of bFGF found in osteocytes was lower than in chondrocytes. Meanwhile, in cleft-affected bone tissue, the relative amount of bFGF was lower than in the control bone tissue, however,

without a statistically significant difference between the groups. Whereas bFGF is necessary for bone remodeling in early bone healing processes (Kawaguchi et al., 2007; Ueno et al., 2011), it is possible that cleft-affected bone tissue, compared to healthy bone tissue, has a lower potential for tissue healing.

In cleft cartilage tissue in the study, the relative number of bFGF was higher compared to the control group, with no statistically significant difference between the groups. An increased number of bFGF positive chondrocytes in the cartilage tissue of cleft-affected patients could indicate the effect of surgical intervention on the ongoing process of tissue healing or remodeling. The increased presence of bFGF in chondrocytes could potentially be considered a positive effect, as it could indicate a more active regenerative process of cleft-affected cartilage tissue and recovery after surgical intervention (Everts et al., 2020).

Statistical analysis suggests that there are no significant differences between the relative number of bFGF for the first time and re-operated cleft patients. This could mean that surgical intervention, whether first-time or repeated, could result in the same release of bFGF. However, when comparing the cleft patient groups with the control groups, differences were found in the distribution of bFGF. This could justify the potentially different role of bFGF in tissue regeneration and repair after surgery between bone and cartilage groups.

TGFβ is a family of pluripotent cytokines consisting of three isoforms: TGFβ1, TGFβ2 and TGFβ3 with TGFβ1 as the dominant growth factor in the wound healing process. After an acute traumatic process, TGFβ1 regulates and secretes keratinocytes, platelets, fibroblasts and macrophages. It is an essential growth factor for the initiation of the inflammatory process and the formation of granulation tissue (Barrientos et al., 2008). TGFβ1 also participates in the process of angiogenesis, regulating the release of vascular endothelial growth factors and facilitating the migration of keratinocytes during

wound closure (Yang et al., 2007). TGFβ1 is an important growth factor in the process of bone formation and remodeling, and studies have shown that TGFβ can stimulate early osteoblast differentiation while inhibiting the differentiation of late osteoblasts in osteocytes (Tang and Allison, 2013).

Our study found no statistically significant difference between cleft bone tissue and the control group, where TGFβ1 was detected in small numbers. It is possible that the relatively small presence of TGFβ1 in bone tissue could have affected the healing process of a cleft bone wound after surgical intervention. Similar data were published in a study where the relatively small amount of TGFβ was found in the epidermis of chronic non-healing wounds (Pastar et al., 2010).

TGFβ affects the entire life cycle of chondrocytes and promotes a range of cellular reactions, including cell survival, proliferation, migration, and differentiation. Since TGFβ is involved in maintaining a balance between chondrogenic differentiation and chondrocyte hypertrophy, its regulatory role is especially important for the development of cartilage. In cartilage tissue, it can contribute to the viability of chondrocytes (Horner et al., 1998), affect chondrogenesis, and help restore damaged cartilage (Choi et al., 2013; Yang et al., 2021). Our study did not reveal statistically significant differences between cleft-affected cartilage tissue and the control group. However, the relative number of TGFβ1 here was greater than in bone tissue, which could indicate a better regeneration capacity of cartilage tissue compared to bone tissue.

MMP-2, also known as gelatinase A, is primarily responsible for the breakdown of collagen types IV and V. MMP-2 expression is significantly involved in a variety of physiological and pathological processes, including embryogenesis (Zhang et al., 2003), tissue remodeling, wound healing, inflammatory process (Parks et al., 2004), and tumor invasion (Deryugina and Ouigley, 2006).

The results obtained in our study and control groups indicated the presence of MMP-2 in all bone and cartilage tissue. The number of positive osteocytes and chondrocytes varied from rare to numerous. Such differences can be explained by the process of internal transformation of bone and cartilage tissue, which is associated with the continuous process of rebuilding.

The relative number of MMP-2 positive osteocytes in our first-time operated patients was rare, which does not indicate a potentially different rebuilding process compared to the control group, where their number was also statistically significantly not higher. Interestingly, the relative number of MMP-2 positive osteocytes differed statistically significantly between first-time and re-operated cleft patients' bone tissue. In particular, re-operated cleft patients showed a significant increase in the number of MMP-2-positive osteocytes, which could indicate increased bone remodeling as a response to repeated surgical intervention and subsequent healing process. A similar increase in MMP-2 was also observed during enhanced wound healing (Hsu et al, 2006). This suggests that after multiple surgical procedures, the processes of bone remodeling and regeneration may become more aggressive or disregulated. The same conclusion was reached by *Henriet* with co-authors in a 2019 study where increased levels of MMP-2 were associated with a poorer prognosis for therapy (Henriet et al., 2019). This is also confirmed by *Dutta* with co-authors, who showed that as the level of MMP-2 increases, its catalytic activity in the bone ECM increases, with subsequent osteoblast dysfunction and bone destruction (Dutta et al., 2014).

In our study, MMP-2 correlated positively with TGFβ1, which could indicate increased bone remodeling and destruction by increasing the resorptive function of osteoclasts (Luis-Ravelo et al., 2014). Similar results are observed in mice with increased expression of TGFβ, whose bone mass and strength decrease (Balooch et al., 2005). Angiogenesis is necessary for the formation of a new bone

in the process of osteogenesis. The proteolytic action of MMP-2 can release and activate vascular growth factors present in the ECM, such as VFGF and bFGF (Cheng et al., 2007). We also observed such data in our study, where a moderate, strong positive correlation with bFGF was found, suggesting the formation of angiogenesis and, potentially, the beginning of the process of osteogenesis.

We found a similar trend in MMP-2 in the cartilage tissue of cleft-affected patients, where MMP-2-positive chondrocytes were detected more than in the control group. This is consistent with the theory that cartilage, much like a bone, degrades and remodels in response to pathological conditions that could be further amplified by surgical interventions. Similar data are present in a 2020 study where an increase in the amount of MMP-2 was observed in patients with repeated knee meniscus surgery (Mull et al., 2020). It should be noted that in our study, no significant differences were observed between the cartilage tissue of first-time operated and re-operated cleft patients, which is likely to indicate a less pronounced cartilage remodeling response to repeated surgical intervention.

However, we found interesting data in the correlation of the relative number of MMP-2 positive chondrocytes with tissue factors. While the cartilage tissue for the first-time operated cleft patients results positively correlated with TIMP2 alone, moderately strong, positive correlations of MMP-2 with TGFβ1, bFGF, IL-1α, IL-10, and HBD-3 were observed in the cartilage tissue of the re-operated cleft patients. This could indicate that the first-time surgery of cartilage still maintains a balance between MMP and TIMP expression, so that there may not be increased cartilage degradation (Tanigawa et al., 2005). Positive correlations observed in the cartilage tissue of repeatedly operated cleft patients, in turn, suggest increased involvement of immune function, as well as a more pronounced wound healing process, as a result of repeated tissue injury (Xie et al., 2020).

MMP-8, also known as neutrophil collagenase, is essential in the breakdown of ECM both in physiological processes such as embryonic development, reproductive process, tissue remodeling, also pathological processes such as inflammation and oncogenesis (Visse et al., 2003; Zucker et al., 2000).

In the literature there is not much data on the effect of MMP-8 on bones and cartilage after an injury. More MMP-8 has been described in relation to various inflammatory diseases of the oral cavity that can occur with bone damage, when higher levels of MMP-8 in saliva correlated with greater damage to the alveolar bone (Al-Majid et al., 2018; Gursoy et al., 2010). For the first time in the group of operated cleft patients, the relative amount of MMP-8 positive osteocytes ranged from rare to numerous. On the other hand, MMP-8 expression was not observed in all reoperated cleft patients. This difference could indicate that surgical intervention affects the expression of MMP-8 in the bone tissue of cleft patients.

A difference in the amount of MMP-8 positive osteocytes was found between the control group and re-operated cleft patients. The presence of MMP-8 was lower in first-time operated cleft patients compared to the control group. In the process of wound healing under inflammatory conditions, neutrophils go to the wound site, releasing MMP-8, which is necessary in the process of healing the wound. Reduced expression of MMP-8 can lead to late healing of the wound (Gutierrez-Fernandez et al., 2007). This suggests that the level of MMP-8 could potentially be affected by surgical intervention, since the expression of this degradation enzyme decreases after surgery, indicating worse wound healing. However, it is worth noting that there was no statistically significant difference in the relative amount of MMP-8 positive osteocytes between first-time surgery and re-operated cleft patients.

When evaluating cartilage tissue, MMP-8 was found in all samples of control and cleft affected patient groups, and the number of positive structures in all groups ranged from numerous to abundant. A more intense expression of MMP-8 compared to bone tissue could indicate an increased process of cartilage remodeling and a possible more intense course in the process of cartilage degradation, coinciding with data from another study on cartilage damage (Cole et al., 1996). Also in this case, the relative number of positive chondrocytes in MMP-8 did not differ between all study groups.

MMP-9, also known as gelatinase B, mainly breaks down elastin, collagens of types IV, V and XI, which are essential in maintaining the structural integrity of tissues and organs (Murphy, 2011). Interestingly, osteoclasts and trophoblasts express MMP-9 early, but later it is mostly secreted by inflammatory cells (Vu and Werb, 2000). MMP-9 is also involved in the process of angiogenesis, migration of cells to the wound site, as well as its epithelisation. Its secretion was stimulated by TGFβ activation (Salo et al., 1994), while increased expression of MMP-8 and MMP-9 contributed to poorer wound healing (Chang et al., 2016).

In patients in the control group, MMP-9 positive osteocytes were observed in all samples. For the first time and in the bones of re-operated cleft patients, MMP-9 positive osteocytes were observed in an overall smaller number than in the control group, however, without a statistically significant difference. Perhaps the relative decrease in MMP-9 positive osteocytes in bone tissue compared to the number of MMP-9 positive chondrocytes in cartilage tissue indicates that wound healing could occur more intensively in bone tissue than in cartilage tissue. A 2011 study showed that loss of MMP-9 improved bone trabecular density (Nyman et al., 2011). To date, the increased expression of MMP-9 has been observed in inflammatory and tumor tissues (Giraudo et al., 2006) compared to healthy tissue (Yousef et al., 2014). In our patients, the trauma

of surgery may not have been pronounced enough in the bone tissue to provoke intense expression of MMP-9. However, MMP-9 is necessary for bone development and healing, as altered ossification as well as late healing of bone fractures were observed in MMP-9 zero mice (Colnot et al., 2003).

For the mechanical strength and remodeling of cartilage, regulated collagen homeostasis is essential, which requires unchanged, balanced expression of MMP (Clark et al., 2008). This balance of MMP can be disrupted by inflammatory diseases (Burrage et al., 2006), mechanical stress or trauma. The latter causes increased expression in tissues of MMP, including MMP-9, which leads to more pronounced cleavage of the components of the cartilage ECM and degradation of cartilage tissue (Mixon et a., 2021). In the cartilage tissue of our study, MMP-9 was found more in patients with cleft than in the control group. A significant difference was observed between the control group in both the first time and the re-operated cleft patients. This means that the operation can lead to increased expression of MMP-9 in cartilage. Likewise, the relative number of MMP-9 in cleft-affected cartilage tissue was moderate to numerous, which may additionally indicate increased ECM cleavage and tissue damage.

TIMP is a group of four proteins (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) that inhibit activated MMP activity (Lambert et al., 2004). The balance between MMP and TIMP is crucial to ensure the proper functioning of the ECM and, consequently, tissue regeneration and remodeling (Wang et al., 2020). TIMP-2 acts on MMP-2 by inhibiting it and regulating its activity (Bernardo et al., 2003). TIMP is able to inhibit bone resorption, which in itself is an important physiological process in bone growth, remodeling and wound healing, but is too pronounced in case of pathologies (Katsunuma, 1997).

When analyzing the TIMP-2 finding, we did not observe statistically significant differences between the study groups. However, the relative number

of TIMP-2 was greater in cartilage compared to bone. A 2019 study found that complete TIMP loss leads to increased MMP activity and altered osteoblast proliferation, mineralisation, and ECM synthesis. In osteoclasts, in turn, increased activity was observed. All these factors lead to bone resorption and loss of bone mass (Chen et al., 2019). In our study, the relative number of bone tissue MMP-2 in re-operated cleft bones was higher compared to the bone tissue of first-time cleft patients, while the expression of TIMP-2 did not differ statistically significantly between the first-tim operated and re-operated cleft patients. To date, data on these tissue factors we studied and their interactions in cleft-affected supporting tissues after repeated surgical interventions have not been published. Therefore, an imbalance in the relative amount balance of MMP-2/TIMP-2, which could indicate increased cleavage, degradation and possible influence on the integrity of the bone under the influence of repeated tissue injury.

The relative number of positive structures of TIMP-2 in cartilage tissue was greater than in bone tissue, while in comparison with the relative number of MMP-2, it was less. Several studies have found that under the influence of cartilage injury or degeneration, changes in TIMP-2 expression are not as pronounced compared to other representatives of the TIMP group (Asik et al., 2020; Zhang et al., 2018). It is important to note that despite the observed differences in the relative amount of TIMP-2 in bone tissue between the study groups, the relative amount of TIMP-2 did not differ between the cartilaginous tissue study groups. This could suggest that while surgical interventions may affect TIMP-2 levels, the role of TIMP-2 in cartilage tissue ECM remodeling remains the same between study groups. Similarly, the TIMP-2 finding in our study correlated statistically strongly with MMP-2 in first-time cleft patients. However, in reoperated cleft patients, TIMP2 correlated statistically significantly moderately strongly with OPG, which could indicate the role of OPG as

a compensatory mechanism in inhibiting cartilage degradation (Kadri et al., 2008). However, a more pronounced expression of MMP and TIMP could indicate a more active remodeling of ECM in cleft-affected cartilage tissue compared to bone tissue, as well as maintaining a balance between tissue degradation and suppression of this process.

IL-1α is a powerful inflammation-promoting cytokine that binds neutrophils and monocytes to the tissue damage site and activates MMP. IL-1α stimulates the production of other cytokines, such as IL-6 and TNF-α, during the inflammatory process (Schett et al., 2016).

In bone, IL-1α enhances osteoclastogenesis, differentiation of osteoclasts and their activity in the bones, which can lead to bone loss (Polzer et al., 2010). IL-1α has also been studied to reduce OPG expression in osteoblasts (Tanabe et al., 2005). In our study, no statistically significant differences were found between all study groups, however, more IL-1α was found in cartilage tissue than in bone tissue. IL-1α is one of the most important inflammation-promoting cytokines associated with cartilage degradation. IL-1α can lead to an increase in the number of catabolic mediators in chondrocytes, most of which are involved in the destruction of cartilage, the promotion of the process of ECM degradation, and the apoptosis of chondrocytes after traumatic damage (Conde et al 2021). The possible increased presence of IL-1α in the cartilage tissue of our patients could indicate a more active process of cartilage damage with the presence of persistent inflammation and a negative impact on the integrity of cartilage tissue. This assumption is implicitly confirmed by data from other authors, namely, excessive expression of IL1-α leads to the development of arthritis, destroying cartilage and bones, while mice that lacked IL-1α receptors did not develop arthritis (Ji et al., 2002; Yi et al., 2018). In addition, these observations coincide with the finding in OA patients, where IL-1α discharge retention improved disease symptoms (Chevalier et al., 2009). On the other hand, the injection of

the IL-1α receptor antagonist caused a reduced number of activated osteoclasts, thus causing a delay in tooth hatching (Meng et al., 2020). In our study, both the low number of IL-1α positive osteocytes in the cleft group and the undetected differences between the cleft and control groups suggest that IL-1α may not be a major factor in the pathogenesis of cleft.

IL-10 is an anti-inflammatory cytokine that inhibits the release of inflammatory factors. T and B lymphocytes secrete IL-10, which regulates the differentiation of osteoblasts. Likewise, it is an inhibitor of the formation of both osteoclasts and osteoblasts through the RANK/RANKL/OPG axis (Moore et al., 2001). Specifically, IL-10 inhibits the differentiation of osteoclasts by increasing the amount of OPG (Liu et al., 2006).

The data from our study showed that bone tissue contains a lower number of IL-10-positive osteocytes compared to IL-10-positive chondrocytes. In the bone group, the number of IL-10 positive osteocytes in first-time cleft patients was lower than in the control group, however, in the re-operated cleft patient group, the number of IL-10 positive osteocytes were no different from the control and the group of cleft patients who had been operated on for the first time. Studies of the effect of IL-10 on bone tissue are mixed. Elevated levels of IL-10 were found to contribute to the osteogenesis of dental pulp stem cells by improving osteogenic differentiation (Yuan et al., 2021), while osteoporosis patients had significantly lower levels of IL-10 than healthy people (Ma et al., 2021). Likewise, mice with IL-10 deficiency had reduced bone mass, increased bone fragility, and inhibited their formation (Dresner-Pollak at al., 2004). Yi et al. showed that increased IL-10 counts reduced osteoblast apoptosis, bone absorption, and decreased TNF-alpha synthesis and excretion (Yi et al., 2018).

It is possible that the reduced number of IL-10 in the tissues affected by the cleft could indicate an imbalance in the course of inflammation and disturbances in the homeostasis of bone tissue, which could potentially worsen

the postoperative recovery process of cleft patients. Bone tissue may react more strongly to a first-time surgical intervention, however, after repeated surgery, tissue adaptation occurs and anti-inflammatory activity returns to the level of normal, however, this is only a hypothesis that should be further proven in the future.

IL-10 in cartilage tissue is known to stabilise the chondrogenic phenotype after cartilage tissue compression and maintain the integrity of the ECM (Behrendt et al., 2018). In addition, IL-10 restores the biosynthetic activity of chondrocytes of damaged joints in the inflammatory environment and improves the synthesis of proteoglycans (Jansen et al., 2008). In our study, the relative number of IL-10 in cartilage tissue was greater than in bone tissue, where a higher number of IL-10 was statistically significantly observed in cleft-affected patients than in the control group.

We found that IL-10-positive chondrocytes formed a strong, statistically significant positive correlation with IL-1α in both the first-time surgery group and the repeated surgery cleft group, which is likely to indicate a compensatory anti-inflammatory protective mechanism in patients' cartilage. These data coincide with a 2013 study by Jin and co-authors, where it was shown that the release of inflammatory cytokines stimulates the release of IL-10, to regulate the immune response, and to prevent excessive inflammation (Jin et al., 2013). IL-10 could be an indicator of a compensatory anti-inflammatory protective mechanism in cleft-affected cartilage tissue, as additionally supplied to IL-10 cartilage, in conditions of artificially inflicted injury, improved chondrocyte differentiation and cartilage ECM formation (Behrendt et al., 2018).

HBD are special proteins, or antimicrobial peptides, that are part of the immune system. They can alter the immune response and are involved in protecting against pathogens by attaching immune system cells and modulating their functions (Fruitwala, et al., 2019). The antimicrobial mechanism of HBD

works through cell destruction by osmolysis (Hoover et al., 2003). Most often, HBD is found in mucous membranes, skin and parts of the body that have contact with potentially harmful substances (Ghosh et al., 2019). In bones and cartilage, the release of HBD is stimulated after inflammation and in the process of tissue regeneration. In order for a bone defect to be corrected, osteogenesis is an essential aspect of the bone restoration process. HBD-2 has been shown to improve osteogenesis by increasing expression of Runx2, OC, and OPN in vitro (Peng et al., 2020). In our study, lower levels of HBD-2 and HBD-3 were observed in bone tissue than in cartilage tissue. Bone infections are one of the main causes of unsuccessful bone graft surgery. HBD-2 embedded in the graft inhibits bacterial growth, and also promotes osteogenic differentiation and bone healing (Ren et al., 2021). In another study, lowered levels of HBD-2 in the human gum epithelium were cited as a possible cause of exacerbation of periodontal disease (Mahanonda et al., 2009). In contrast, the expression of HBD-3 mRNA after treatment of osteonecrosis of the jaw was increased, suggesting that HBD could be an indicator of the effectiveness of the treatment of bone pathologies (Thiel et al., 2020). The low level of IL-10, HBD-2, and HBD-3, and the relative number of positive osteocytes in the bone tissue of cleft-affected patients could indicate a tendency to a reduced anti-inflammatory mechanism and bone recovery after primary and repeated surgical interventions.

However, HBD-2 showed no statistically significant differences in cartilage tissue comparator groups, while HBD-3 was observed in greater quantities in patients in both cleft groups than in the control group. There are not many studies showing the effect of increased HBD-3 on cartilage tissue, but studies on wound healing, namely after treatment with HBD-3, wounds healed faster and contained an increased number of fibroblasts and blood vessels (Takahashi et al., 2021). The formation of HBD-3 can also be induced without exposure to bacteria. TNF-α and IL-1 are powerful inducers of HBD-3.

The application of HBD-3 on cartilage leads to increased production of TIMP-1 and TIMP-2. This could be indicative of HBD-3's multifunctional ability to bind defense mechanisms and inflammation through tissue remodeling processes (Varoga et al., 2005). HBD-3 is thought to be involved in the process of remodeling articular cartilage tissue, and although HBD-3 has anti-inflammatory properties, it has been shown to have a positive correlation with inflammatory process and inflammation-promoting cytokines such as TNF-α and IL-1β (Mohammed et al., 2023).

The results of our study may suggest that although the relative number of HBD was higher in cartilage tissue than in bone tissue, which could indicate better protective abilities of cartilage tissue after injury, it is nevertheless possible that the increased expression of HBD-3 could lead to increased involvement of the immune system, which could be the cause of tissue damage. However, the increased relative number of IL-10 and the differences found in the relative quantities of IL-1α in the tissues between the cleft and control groups allow for better antimicrobial and anti-inflammatory properties of cartilage tissue.

Runx2 belongs to the Runx gene family and is a transcription factor. This is necessary for the correct placement of osteoblast cells (Dos Santos Pereira et al., 2017). Runx2 promotes bone formation, mineralisation, osteoblast proliferation and induces the differentiation of mesenchymal cells in immature osteoblasts to the final stage of this process, in which osteoblast maturation is inhibited (Komori, 2010). Likewise, Runx2 induces the hypertrophy-related genus of many chondrocytes and positively regulates the hypertrophic differentiation and ossification of chondrocytes (Nagata et al., 2022).

Our study found no differences in Runx2 positive chondrocytes and osteocytes between the study groups. However, in bone tissue, Runx2 positive osteocytes were rarely observed, while Runx2 positive chondrocytes were observed in moderate numbers. This could potentially indicate the problematic

formation of osteoblasts and possibly also reduce the mineralisation potential of bone and cartilage. Similar results were also found in Qin et al. in a 2018 study where the exclusion of Runx2 functions negatively affected the formation of osteoblasts, potentially leading to impaired dental eruption (Qin et al., 2018). The same results were reported in Stricker et al., where a decrease in endochondral ossification was observed in embryos of Runx2 hens due to a delayed process of chondrocyte maturation (Stricker et al., 2002).

Wnt3a is a signalling molecule that belongs to the protein family of the Wnt gene and is included in various skeletal development processes, including stem cell differentiation and migration processes (Logan et al., 2004). Wnt3a is a powerful promoter of bone regeneration, as it improves the genesis of osteoblasts and thus bone formation. In addition, Wnt is known to influence bone resorption by reducing osteoclast activity, modulating osteoclast stem cell differentiation and the RANKL/OPG axis (Wang et al., 2018). In turn, the inactivation of the Wnt signaling pathway leads to ostepenic states (Wagner et al., 2020). Our study found no statistically significant differences between study groups in the relative quantity of Wnt3a. However, low numbers of Wnt3a-positive osteocytes were observed in bone tissue, while numerous Wnt3a-positive chondrocytes were found in cartilage tissue.

These results of the relative number of Wnt3a justify the presence of rare Runx2 positive osteocytes in bone tissue, since the Wnt signaling pathway causes endogenous expression of the Runx2 gene in mesenchymal cells that later participate in bone formation (Gaur et al., 2005). The positive effect of Wnt3a applications on osteogenesis and angiogenesis at the site of bone damage has been demonstrated (Wagner et al., 2020). Thus, an insufficient amount of Wnt3a in bone tissue could indicate poorer bone healing, as well as its association with Runx2 could indicate a reduced mineralisation potential in cleft-affected bone tissue.

Recent studies have shown that Wnt3a may promote cartilage regeneration and the healing of osteochondral defects (Thomas et al., 2121). Excessive activation of Wnt signals leads to loss of cartilage ECM (Loughlin et al., 2004), but still, factor expression at the site of injury is necessary for successful cartilage tissue regeneration (Cheverud et al., 2014). In our study, the relative number of Wnt in cartilage tissue was greater than in bone tissue, which could indicate a higher potential for cartilage tissue regeneration.

For cell death in physiological and pathological conditions, three different types are distinguished: apoptosis, autophagia and necrosis. Unlike necrosis, in the process of apoptosis and autophagia, cytoplasmic membranes are preserved, and the cell dies without an inflammatory process (Comori et al., 2013). Disruption of the regulation of apoptosis leads to pathological tissue conditions such as cancer, developmental abnormalities, and degenerative diseases. **Apoptosis** plays a crucial role in maintaining homeostasis of various tissues, as well as in regulating the normal development of the embryo. After a tissue injury, various cells, including neutrophils, macrophages, and lymphocytes, migrate in its place to initiate tissue regeneration. After the healing process is over, apoptosis begins in some of these cells, which prevents excessive inflammation and resulting tissue damage (Greenhalgh et al., 1998). Also, in osteoblasts or chondrocytes, apoptosis can be caused by physiological or pathological factors (Izu et al., 2011; Liu et al., 2007). Lack of IL-1 can lead to an increase in osteoclast apoptosis with a decrease in the concentration of prostaglandins or other anti-apoptotic factors. TGFβ is able to increase osteoclast apoptosis (Manolagas et al., 2000). Apoptotic osteocytes, in turn, send signals to osteoclasts to initiate targeted bone resorption, which can lead to an improvement in the mechanical properties of the bone (Bellido et al., 2013).

Our results showed that groups of patients affected by cleft bone tissue had lower numbers of apoptosis positive osteocytes than the control group.

The small relative number of IL-1α and the increased number of OPN compared to the control group and its positive correlation with apoptosis coincide with data from other studies (Liu et al., 2007). It is possible that the reduced number of apototic cells in the cleft-affected bone tissue could indicate insufficient destruction of inflammatory cells and, as a result, an increase in the inflammatory process in the bone tissue, as well as a decrease in the resorption capacity of the bone.

The death of chondrocytes and the loss of ECM form a correlation between the degree of cartilage damage and chondrocyte apoptosis. Since apoptosis is usually a fast process, a high rate of apoptosis in cartilage would theoretically result in ECM degradation in a short period of time (Hwang et al., 2015). The results of our study showed a reduced number of apototic cells in re-operated cleft-affected patients compared to the control group as well as to the first-time operated group of patients. These data could indicate the effects of trauma and impaired wound healing after repeated surgery.

OPG, MMP-8, Runx2, and apoptosis positive chondrocytes were the most observed in the cartilage tissue control group. The first-time operated cleft-affected patients had an increased number of OC, OPN, OPG, BMP-2/4 bFGF, TGFB1, MMP-2, MMP-8, MMP-9, TIMP-2, IL-1a, IL-10, HBD-2, Wnt-3a, and apoptosis positive chondrocytes. In re-operated cleft patients, the number of these above factors was increased, as well as the number of HBD-3 was increased. An increase in the relative number of positive cells of these factors, in comparison with the control tissues, as well as the amount of these factors, indicates the intensification of all processes (mineralisation, growth, local protection, remodeling).

OC, BMP-2/4, MMP-8, MMP-9, IL-10, HBD-2, and apoptosis positive osteocytes were observed in the bone tissue control group. A decrease in the relative number of all factors was observed in the bone tissue of patients

affected by the first-time cleft surgery, the highest number of which was found to be OC. From bone tissue of re-operated cleft-affected patients, the relative number of MMP-2 positive cells increased to the level of control tissue, and OC and apoptosis positive osteocytes were observed in larger quantities, which did not reach the level of the relative number of positive cells of the control tissue. This means that the processes of degradation and mineralisation prevail in the bone tissue affected by the cleft.

Conclusions

1. Osteons of various sizes, ingrown connective tissue into Haversian channels, heterogeneous staining, and irregular size of bone tissue plates in cleft patients indicate nonspecific bone changes in cleft bones. The compliance of cartilage tissue with the generally accepted norm justifies the greater strength of these supporting tissues for the cleft process. The lack of changes in the bone and cartilage tissues of cleft patients in first-time and re-operated patients indicates a lack of effect of surgical trauma on these tissues at the routine microscopy level.
2. Healthy facial cartilage tissue is characterised by moderately pronounced OPN, BMP-2/4, bFGF, MMP-2, IL-10, HBD-2, -3, Runx2; pronounced positive structures of TGFβ1, TIMP-2, IL-1α, Wnt3a, apoptosis and especially pronounced OC, indicating active remodeling, growth, excretion and mineralisation of these supporting tissues. The phenotype of healthy bone tissue is characterised by a much smaller presence of OC, MMP-8 and HBD-2, which indicates mainly the main role of individual factors in bone degradation, protection and mineralisation.
3. The cartilage tissue of first-time operated cleft-affected patients reveals increased OPN, bFGF, MMP-2 and -9, IL-10 and HBD-3, as well as the number of positive structures of apoptosis, indicating a selective process – activation of cartilage homeostasis (OPN), growth stimulation (bFGF), increased degeneration (MMP-2), also stimulation of local protection (IL-10 and HBD-3), as well as the promotion of programmed cell death in case of surgical correction of tissues. In re-operated cartilage tissue, the increased number of BMP-2/4 structures also join the above phenotype, suggesting an even more intense growth of corrected cartilage tissue.

4. First-time operated bone tissue in cleft-affected patients is characterised by increased OPN but decreased OC, bFGF, BMP-2/4, IL-10, HBD-2, and -3, and apoptosis positive cells, suggesting activation of plastic-affected bone homeostasis under conditions of reduced mineralisation, growth, local protection, and programmed cell death. However, the constant amount of OPG, MMPs, TIMP-2, IL-1α, and gene proteins justifies maintained suppression of osteoclastogenesis, tissue remodeling, and tissue resorption balance. In the bone tissue of repeatedly operated cleft patients, an increase in the number of positive structures of BMP-2/4 and IL-10 to the level of control tissue, also an increase in MMP-2, but a decrease in MMP-8, indicates a selective return of bone growth and protection factors to the normal level, but selective action of MMP with the activation of the main cell MMP – MMP-2.
5. OPN, BMP-2/4, bFGF, IL-1α, and HBD-3 were most often correlated with each other in the cartilage tissue of first-time operated cleft patients, while TGFβ1, MMP-2, IL-1α, and IL-10 were most often correlated with each other in the cartilage tissue of reoperated cleft patients. These correlations confirm the association of more intense cartilage selective growth, protection, and homeostasis regulation factors in cleft patients against the background of constant bone resorption rates.
6. For the first time, the most common correlations in the bone tissue of operated cleft patients were found with OPN, BMP-2/4, bFGF, TIMP-2, IL-1α, IL-10, HBD-2, and Wnt3a. In the bone tissue of repeatedly operated cleft patients, bFGF, MMP-8, IL-1α, IL-10, and HBD-2 were most often correlated with each other. These correlations support the relationship between bone growth-stimulating, local protection, and resorption-stimulating factors in cleft-affected patients, but selectively establishing correlations between bone growth, homeostasis, factors contributing to cell proliferation more after

first-time surgeries. In repeatedly affected bone tissue, a decrease in total correlations indicates a possible imbalance in bone tissue function, which is compensated by the intensification of the action of protection factors and reduced by the intensification of the action of degradation factors.

Proposals for research in the future

The tissue factors considered in this study are only part of the known factors involved in the morphopathological processes of facial clefts. Further study of these and other factors could be a possible direction in the study of the morphology of facial clefts.

Comparing tissue factors after first and repeated maintenance surgeries could be an important direction to help adjust and develop cleft surgeries in the future with better outcomes from invasive procedures and surgery. Further study of BMP-2/4, bFGF, MMP-2, IL-1α, IL-10, HBD-2 and HBD-3 would be an important direction in the study of the factors applied in the clinic.

The study of facial cleft-affected support tissues at the gene level by *the in situ* hybridisation method, as well as the study of genes, growth factors and cytokine concentrations by the ELISA method, would be potential prospective morphological studies in the future.

List of publications, reports and patents on the topic of the Thesis

Publications included in data bases: Scopus, Web of Science, Pubmed:

1. **Buile, D**., Pilmane, M., & Akota, I. (2022). Evaluation of the multiple tissue factors in bone of primary osteoplasty and rhinoplasty in patients affected by cleft lip palate. Histology and Histopathology, 37(7), 679–690. Article 18451. https://doi.org/10.14670/HH-18-451

2. **Buile, D**., Pilmane, M., & Akota, I. (2022). Evaluation of the Multiple Tissue Factors in the Cartilage of Primary and Secondary Rhinoplasty in Cleft Lip and Palate Patients. Pediatric Reports, 14(4), 419–433. https://doi.org/10.90/pediatric14040050

3. **Buile, D**., Pilmane, M., & Akota, I. (2020). Appearance and distribution of tissue remodellation factors in the hard tissue of patients affected by cleft lip palate. Proceedings of the Latvian Academy of Sciences, Section B: Natural, Exact, and Applied Sciences, 74(3), 171–180. https://doi.org/10.2478/prolas-2020-0028

Reports and theses at international congresses and conferences:

1. **Buile, D.,** Pilmane, M., & Akota, I. (2024). Evaluation of 17 morphopathological tissue factors in cartilage and bone after first time and repeated osteoplasty and rhinoplasty in patients with cleft lip and palate. In P. Fedirko, M. Pilmane, T. F. Babenko, & N. Garkava (Eds.), Practical Ophthalmology. Medical and Environmental Problems of our Days: Collection of Works International Scientific and Practical Interdisciplinary Conference (pp. 11–12). State Institution «National Research Center for Radiation medicine of the National Academy of Medical Sciences of Ukraine".

2. **Buile, D.,** Pilmane, M., & Akota, I. (2023). Evaluation of 17 morphopathological tissue factors in cartilage for first-time rhinoplasty or rhinoplasty performed after osteoplasty in patients with cleft lip and palate. In P. Fedirko, M. Pilmane, O. Maksymuk, T. F. Babenko, & N. A. Garkava (Eds.), Practical Ophthalmology. Medical and Environmental Problems of our Days : Collection of Works International Scientific and Practical Interdisciplinary Conference (pp. 7–9). State Institution «National Research Center for Radiation medicine of the National Academy of Medical Sciences of Ukraine".

3. **Buile, D**., Pilmane, M., & Akota, I. (2023). Evaluation of various tissue factors in the bone of first and second time performed surgery (osteoplasty and rhinoplasty) in cleft lip palate patients. Medicina (Kaunas), 59(Suppl. 2), 553.

4. **D. Buile**, M. Pilmane, I. Akota ‘’Characterisation of OPN, OC, ßdef-2, bFGF and MMP-2 in bone of cleft lip palate (CLP) patients from first time plastic alveolar osteoplasty ‘’16th joint symposium of Rostock University and Rīga Stradiņš University and 10th congress of Baltic Association for Maxillofacial and Plastic Surgery 03–04 June 2022, pp 31.

5. **Buile, D**., Pilmane, M., & Akota, I. (2022). Evaluation of the multiple tissue factors in cartilage of performed primary and secondary rhinoplasty in patients affected by cleft lip palate. Anatomy, 16(Suppl.2), S168. https://dergipark.org.tr/tr/download/article-file/2870381

6. **Buile, D.,** Pilmane, M., & Akota, I. (2021). Morphopathogenic aspects of tissue factors in bone of primary osteoplasty and rhinoplasty in cleft lip palate patients. 423. Abstract from RSU Research week 2021: Knowledge for Use in Practice, Rīga, Latvia.

7. Pilmane, M., Jain, N., **Buile, D**., Akota, I., & Kroiča, J. (2021). The concentrations of different cytokines in the cleft lip palate affected tissue. 425. Abstract from RSU Research week 2021: Knowledge for Use in Practice, Rīga, Latvia.

8. **Buile, D**., Pilmane, M., & Akota, I. (2019). Characterisation of OPG, TGF-ß, Runx2 and Wnt3a in Cleft Lip Palate (CLP) Hard Tissue from First Surgical Intervention. 569. Abstract from RSU International Research Conference 2019, Riga, Latvia.

9. **Buile, D**., Pilmane, M., & Akota, I. (2019). Morphopathogenetic aspects of cleft lip palate affected tissue. Medicina (Kaunas), 55(Suppl.2), 231.

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