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Mārtiņš Vaivads

Investigation of Genes and Gene Proteins in Cleft Affected Tissue

Summary of the Doctoral Thesis for obtaining the scientific degree "Doctor of Science (*PhD*)"

Sector Group – Medical and Health Sciences
Sector – Basic Medicine
Sub-Sector – Histology and Cytology

Riga, 2023

Mārtiņš Vaivads

ORCID 0000-0001-8282-9733

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The Doctoral Thesis was developed at Institute of Anatomy and Anthropology of Rīga Stradiņš University, Department of Morphology, Latvia

Supervisors of the Doctoral Thesis:

Dr. med., Dr. habil. med., Professor **Māra Pilmane**,
Rīga Stradiņš University, Latvia

Dr. med., Professor **Ilze Akota**,
Rīga Stradiņš University, Latvia

Official Reviewers:

Dr. med., Professor **Ilze Štrumfa**,
Rīga Stradiņš University, Latvia

Dr. med. vet., Professor **Ilmārs Dūrītis**,
Latvia University of Life Sciences and Technologies

Dr. med., Professor **Ingrida Balnytė**,
Lithuanian University of Health Sciences

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Secretary of the Promotion Council:

Dr. med., Acting Assistant Professor **Ģirts Šalms**

Table of Contents

Abbreviations used in the Thesis 5

Introduction 6
Aim of the Thesis 9
Tasks of the Thesis 9
Hypothesis of the Thesis 10
Novelty of the Thesis 10

1 Materials and methods 11
1.1 Material used for the morphological study and the characterization of patient groups 11
1.2 Morphological methods 12
1.2.1 Tissue fixation 12
1.2.2 Routine histology staining method 13
1.2.3 Immunohistochemistry method and reagents 14
1.3 Data processing methods 17
1.3.1 Semiquantitative counting method 18
1.3.2 Statistical methods 18

2 Results 20
2.1 Morphological findings 20
2.2 Immunohistochemical characterization of cleft candidate genes 21
2.2.1 BARX1 gene protein 21
2.2.2 DLX4 gene protein 22
2.2.3 FOXE1 gene protein 24
2.2.4 HOXB3 gene protein 26
2.2.5 MSX2 gene protein 28
2.2.6 PAX7 gene protein 30
2.2.7 PAX9 gene protein 32
2.2.8 RYK gene protein 34
2.2.9 SHH gene protein 36
2.2.10 SOX3 gene protein 38
2.2.11 WNT3A gene protein 40
2.2.12 WNT9B gene protein 41
2.3 Data about correlations between immunohistochemically determined cleft candidate genes 43

3 Discussion 48

Conclusions 70

Proposals for research in the future 72

List of publications, reports and patents on the topic of the Thesis 73
References 75
Acknowledgments 83

Abbreviations used in the Thesis

AAI	Institute of Anatomy and Anthropology
BARX1	BarH-like homeobox 1
BMP	Bone morphogenetic proteins
DLX	Distal-less homeobox
DLX4	Distal-less homeobox 4
FOXE1	Forkhead box E1
HOX	Homeobox genes
HOXB3	Homeobox B3
IMH	Immunohistochemistry
MSX1	Muscle segment homeobox 1
MSX2	Muscle segment homeobox 2
PAX3	Paired box transcription factor 3
PAX7	Paired box transcription factor 7
PAX9	Paired box transcription factor 9
RYK	Receptor-like tyrosine kinase
r_s	Spearman correlation coefficient
RSU	Rīga Stradiņš University
SHH	Sonic hedgehog
SOX3	SRY-box transcription factor 3
TGFβ	Transforming growth factor beta
WNT	Wingless-type signalling
WNT3A	Wingless-type MMTV integration site protein 3A
WNT9B	Wingless-type MMTV integration site protein 9B

Introduction

Orofacial clefts are congenital developmental defects that are caused by defective fusion of the growing facial folds. Orofacial clefts can have multiple phenotypes and variations, which affects their localization and clinical severity (Dixon et al., 2011). These defects of the craniofacial region can cause significant clinical manifestations that affect the following development of the child, the formation of speech skills, cause respiration and swallowing disturbances, affect the development of sense of hearing and olfaction, while also creating an aesthetic defect and increase the socioeconomic burden for families of cleft patients (Wehby and Cassell, 2010). Facial clefts globally are one of the most common congenital pathologies with a global incidence of 1/700 newborns (Dixon et al., 2011).

The proper development process of the facial region requires interaction between different genes and their coded proteins. The different location, activation, and impact assessment of these regulatory factors in the creation of craniofacial region are essential to fully understand the morphopathogenesis of different clefts in human tissues.

BarH-like homeobox-1 (*BARX1*) is a gene expressed in ectomesenchyme cells of the upper and lower jaw during the facial development process, regulating the condensation of bone tissue and cartilage cell precursors (Welsh et al., 2018). In animals, reduced expression of *BARX1* causes defects in the process of osteochondrogenic cell condensation and hypoplasia of the upper jaw (Shimomura et al., 2019).

Distal-less homeobox (*DLX*) genes are expressed in certain stages of the body's development and provide the formation of the body axis and the development of virtually all organ systems (Trinh et al., 2015a). The clinical manifestations of *DLX* gene disorders have not been well studied in humans, but a variant of the *DLX4* gene has been associated with bilateral cleft lip and palate

formation (Wu Q. et al., 2015). DLX genes are also important in the process of facial development, and their proteins can be found in the ectomesenchyme of the maxillary and mandibular folds (Jeong et al., 2012).

Forkhead box protein E1 (FOXE1) is a transcription factor that enables the formation of multiple organs and their structure during embryogenesis, including the development of the palate and participates in the formation of secondary palate and the fusion of maxillary and frontonasal folds (Dathan et al., 2002). Several mutations in the *FOXE1* gene have been linked to the development of cleft palate and cleft lip (Moreno et al., 2009).

Homeobox genes (*HOX*) encode transcription factors that regulate cell differentiation and proliferation in embryogenesis, ensuring proper organ system development. The homeobox B3 (*HOXB3*) gene regulates the migration of neural crest cells, which is essential for the development of the pharyngeal arch region, including the development of the face (Frisdal and Trainor, 2014).

Muscle segment homeobox 2 (MSX2) is involved in the head development process, where it regulates the proliferation of the preosteoblasts in the head region and the products of this gene can be found within the orofacial area: mandible, Meckel cartilage, and tooth buds (Alappat et al., 2003). Mutations in the *MSX1* and *MSX2* genes are associated with the development of different cleft palate and cleft lip phenotypes (Dai et al., 2014).

Paired box transcription factor 7 (PAX7) and paired box transcription factor 9 (PAX9) are transcription factors that influence the development of neural crest cells and the palate (Sull et al., 2009). PAX7 can be found in palatal shelves, Meckel's cartilage, and epithelium of the nasal cavity (Leslie and Marazita, 2013). Studies in mice have found that mutations in this gene are associated with abnormalities in the development of the nose and upper jaw, including clefts (Leslie and Marazita, 2013). Meanwhile, the deletion of the

PAX9 gene in mice causes a complete cleft palate, defective tooth and pharyngeal pouch development (Li et al., 2017).

Receptor-like tyrosine kinase (RYK) is a protein involved in the development of craniofacial structures (secondary palate) and neuronal differentiation (Halford et al., 2000). *RYK* mutations in mice cause specific changes in facial phenotype, limb shortening, feeding disorders, and respiratory complications (Halford et al., 2000).

The Sonic hedgehog (*SHH*) gene is critically needed for the face and the head development process. It participates in the formation of the palate and nose and can be found primarily in areas with epithelial-mesenchymal interactions, where it induces the proliferation of mesenchymal cells (Hu et al., 2015).

SRY-box transcription factor 3 (SOX3) inhibits the function of proneural differentiation factors and affects the development of the neural tube and placodes in the head. The role of this factor in the development of clefts has been relatively little studied (Bylund et al., 2003).

The genes of the Wingless-type MMTV integration site (WNT) family encode multiple proteins that provide autocrine and paracrine regulation in the facial development process through the WNT signalling pathway (Chiquet et al., 2008). The *WNT3A* gene provides regional specification of the face in the upper lip, primary, and secondary palate during the development process and is considered one of the candidate genes for cleft lip and palate (Andrade Filho et al., 2011). Meanwhile, deletion of the *WNT9B* gene can cause a cleft lip in mice, with or without a cleft palate (Juriloff et al., 2006).

Facial clefts can be corrected and treated through surgery, but this can have potentially disruptive effects on the further growth process of the face and jaws, which can mean the need for more than one operation per patient (Shi and Losee, 2015). Currently available information on genes and gene products during cleft formation is limited and is evaluated mainly in animal models.

Morphological research of genes and gene products present in human tissue affected by clefts has been conducted for individual growth factors and genes, but not in combined studies evaluating multiple genes and gene products in tissue affected by clefts. Due to the complex acquisition of patient tissue material and ethical considerations, there is a lack of data on changes in the endotype of human cleft tissue determined by genotype tissue, which could potentially provide a better understanding of cleft morphopathogenesis and could provide an opportunity to improve existing therapy methods.

Aim of the Thesis

To determine the distribution and the relative number of cleft candidate gene and their coded protein-containing cells in different types of cleft affected tissue.

Tasks of the Thesis

The following tasks have been proposed for reaching the aim of the Thesis:

1. To determine the routine histological findings in control tissue and cleft affected tissue.
2. To determine the immunohistochemical distribution of cleft candidate gene proteins (BARX1, DLX4, FOXE1, HOXB3, MSX2, PAX7, PAX9, RYK, SHH, SOX3, WNT3A and WNT9B) in control tissue and cleft affected tissue.
3. To determine which changes in the distribution of cleft candidate gene protein are characteristic of unilateral clefts.
4. To determine which changes in the distribution of cleft candidate gene protein are characteristic of bilateral clefts (clinically more severe phenotype).

5. To determine which cleft candidate gene proteins are characteristic for all evaluated cleft phenotypes.
6. To determine the most significant correlations between the evaluated gene proteins in the cleft affected tissue.

Hypothesis of the Thesis

The distribution of cleft candidate genes and their encoded proteins in cleft patients qualitatively differs from the tissue of the control patients.

Novelty of the Thesis

This research work is the first in which the distribution of different cleft candidate gene proteins within human facial cleft affected tissue has been investigated. By using the immunohistochemical method, the relative number of BARX1, DLX4, FOXE1, HOXB3, MSX2, PAX7, PAX9, RYK, SHH, SOX3, WNT3A and WNT9B immunoreactive cells in different cleft affected tissue types has been determined. The complex investigation of cleft candidate gene proteins of this combination has not been done before. Previous research of cleft candidate genes mostly used animal models and animal tissue, which allows us to regard the human tissue material in this Thesis as a unique contribution to the field of cleft candidate gene research. In this research work, cleft tissue phenotypes and possible diagnostic and prognostic gene proteins have been determined, which could be useful for clinical practise.

1 Materials and methods

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

Orofacial cleft tissue material was obtained from the Institute of Stomatology of Rīga Stradiņš University (RSU) at the Cleft Lip and Palate Centre between 2003 and 2020. The soft tissue of the cleft lip and cleft palate was collected during primary lip plastic surgery and soft palate plastic surgery. For complete unilateral cleft lip and palate primary lip plastic surgery was performed in accordance with the Millard method (Millard, 1964) and for bilateral cleft lip modification of the Veau method was used (Lehman et al., 1990). Soft palate plastic surgery was performed using the vomer flap method (Robin et al., 2006).

For the application of routine histological and immunohistochemical methods, the tissue material under study was processed in the Laboratory of Morphology of RSU Institute of Anatomy and Anthropology (AAI). Patients with nonsyndromic complete cleft lip and palate were divided into 3 groups. Lip tissue was obtained from 36 patients with unilateral cleft lip, forming the first group of patients, aged 3 months to 1 year and 5 months. The second group consisted of 13 patients with bilateral cleft lip, aged between 4 months and 1 year and 5 months. The third group consisted of soft cleft palate tissue obtained from 26 patients, aged 8 months to 1 year and 3 months. A total of 75 tissue samples were evaluated.

Some of the control tissue material was obtained during the plastic surgery of the upper lip frenulum, which was collected in year 2013 from children at the SIA “RSU Institute of Stomatology”, forming the first control group. This control group consisted of 7 patients aged 8 to 14 years. The upper lip frenulum tissue material was used in the first control group to evaluate the

immunoreactivity of BARX1, DLX4, FOXE1, HOXB3, MSX2, PAX7, PAX9, and RYK proteins and to compare it with the patient tissue groups. No harm to patients' health was done during the tissue acquisition process. As the soft control tissue material available was very limited and insufficient for the evaluation of all gene proteins, additional lip tissue material was collected from the historical collection of the Institute of Anatomy and Anthropology at Rīga Stradiņš University to form the second control group. The second control group consisted of foetal and neonatal lip tissue material used for immunohistochemical evaluation and comparison of SHH, SOX3, WNT3A and WNT9B immunoreactivity with the patient groups.

1.2 Morphological methods

1.2.1 Tissue fixation

The overnight fixation of tissue with the Stefanini solution (Stefanini et al., 1967) was performed at the RSU Institute of Stomatology's Cleft Lip and Palate Centre immediately after tissue samples were collected during lip plastic and soft palate plastic surgeries. Tissue material after fixation was taken to the RSU AAI Laboratory of Morphology, where further tissue processing was carried out.

The fixed tissue material was dehydrated using alcohol solutions at increasing concentrations (70 ° to 96 °). Degreasing was carried out in the xylol solution, then the tissue was held in paraffin I for one hour and in paraffin II for two hours. The tissue was then imbedded in molten paraffin, creating paraffin blocks in special cassettes where paraffin was poured through a dispenser. Paraffin blocks were used to make 3–4 μm thin tissue cuts using a semiautomatic rotational microtome (Leica RM2245, Leica biosystems Richmond Inc., United States of America). The prepared tissue sections were placed on slides

(HistoBond ® +, Paul Marienfeld GmbH & Co. KG, Germany). The slides with tissue sections were then placed in a thermostat to dry at 56 °C for 20–60 minutes.

Further steps and treatments were performed according to the routine histological method of staining and immunohistochemistry.

1.2.2 Routine histology staining method

Routine tissue histological staining with haematoxylin and eosin was performed to obtain the necessary morphological look for the slide review. The tissue sections, which had been dried in a thermostat and placed on slides, were deparaffinized in xylol. Further rehydration of the tissue sections with gradually lower alcohols was performed. Subsequent staining of tissue cuts with haematoxylin (code 05-M06002, Mayer's haematoxylin, Bio OPTICA Milano S.p.A., Italy) and eosin (code 05-B10003, alcoholic solution of Eosin Y, Bio OPTICA Milano S.p.A., Italy) was performed. After staining, rinsing with running water followed by further dehydration with increasing concentrations of alcohol solutions and clarification with xylene and carboxyxylene. Then histological glue (code 6900002, Paul Marienfeld GmbH & Co. KG, Germany) was applied to the slides and the samples were covered with cover glasses (Carl Roth GmbH + Co, Germany) (Lillie et al., 1976; Feldman and Wolfe, 2014).

In the resulting microspecimens, the basophilic structures were stained blue-violet, and the acidophilic structures stained pink. The preparations stained in haematoxylin and eosin were analysed with a light microscope (Leica DM500RB, Leica biosystems Richmond Inc., United States of America) and treated with the Image-Pro Plus computer programme. The microphotographs of tissue samples were captured with a digital camera (Leica DC 300F, Leica Microsystem Aga, Germany).

1.2.3 Immunohistochemistry method and reagents

Using the biotin-streptavidin immunohistochemical method (Hsu et al., 1981), these cleft candidate genes were determined in tissue:

- **BarH-like homeobox-1** (BARX1, code LS-C29810, derived from rabbit, work dilution 1:100, LifeSpan Biosciences, Inc., United States of America);
- **distal less homeobox-4** (DLX4, code orb160775, derived from rabbit, working dilution 1:100, Biorbyt Ltd., United Kingdom);
- **forkhead box E1** (FOXE1, code ab5080, derived from goat, working dilution 1:500, Abcam, United Kingdom);
- **homeobox B3** (HOXB3, code sc28606, derived from rabbit, work dilution 1:100, Santa Cruz biotechnology, United States of America);
- **muscle segment homeobox-2** (MSX2, code ab223692 obtained from rabbit, working dilution 1:100, Abcam, United Kingdom);
- **paired box transcription factor-7** (PAX7, code ab55494 obtained from mouse, work dilution 1:100, Abcam, United Kingdom);
- **paired box transcription factor-9** (PAX9, code orb11242, derived from rabbit, work dilution 1:100, Biorbyt Ltd., United Kingdom);
- **receptor-like tyrosine kinase** (RYK, code orb38371, derived from rabbit, working dilution 1:100, Biorbyt Ltd., United Kingdom);
- **sonic hedgehog protein** (SHH, code LS-C49806, derived from rabbit, work dilution 1:100, LifeSpan Biosciences, Inc., United States of America);

- **SRY-box transcription factor-3** (SOX3, code orb158460 derived from rabbit, working dilution 1:100, Biorbyt Ltd., United Kingdom);
- **wingless-type MMTV integration site protein-3A** (WNT3A, code ab19925, derived from rabbit, working dilution 1:800, Abcam, United Kingdom);
- **wingless-type MMTV integration site protein-9B** (WNT9B, code ab151220, derived from rabbit, working dilution 1:100, Abcam, United Kingdom).

The fixation of the samples, preparation of the tissue material for placement in paraffin blocks and the further preparation of tissue sections on slides were implemented following the scheme explained in the previous chapter (see Chapter 1.2.1). The tissue sections found on slides that were dried in a thermostat were deparaffinized in xylol. This was followed by dehydration in gradually lower concentration alcohols. The tissue was placed in the holder after deparaffinization for rinsing with TRIS buffer solution (code 2017X12508, Diapath S.p.A., Italy) 2 times for 5 minutes, followed by boiling in EDTA buffer (code 2017X02239, Diapath S.p.A., Italy) in microwave for 20 minutes. After being cooled, the samples were washed twice over 5 minutes with TRIS buffer solution. To block endogenous peroxidase activity, a 3 % peroxide solution was administered to tissue samples for 10 minutes followed by flushing with a TRIS buffer solution 2 times over 5 minutes. Blocking serum was applied to tissue samples for 20 minutes to reduce background colouration. All tissue samples to be analysed were incubated with primary antibody for one hour. All antibodies used in the study were diluted with an antibody diluent (code 938B-05, Cell MarqueTM, United States of America).

Antibodies of mouse or rabbit origin were subjected to the HiDef DetectionTM HRP polymer staining system (code 954D-30, Cell MarqueTM, United States of America) staining system. After incubation with the primary

antibody and triple rinsing, a HiDef DetectionTM reaction amplifier (code 954D-31, Cell MarqueTM, United States of America) was applied at room temperature for 10 minutes. The preparations were then rinsed 3 times for 5 minutes with TRIS buffer solution followed by the addition of the HiDef DetectionTM HRP polymer marker (code 954D-32, Cell MarqueTM, United States of America) and incubated at room temperature for 10 minutes. After incubation, the samples were rewashed 3 times over 5 minutes with TRIS buffer solution. The tissue was then coated with the DAB substrate kit chromogenic system (code 957D-60, cell MarqueTM, United States of America) and incubated at room temperature for up to 10 minutes to obtain the brown colouration of the immunopositive structures.

Antibodies of goat origin were subjected to the ImmunoCruz TM ABC staining system (code SC-2023, Santa Cruz Biotechnology, Inc., United States of America) staining system. Using the ABC staining system, the preparations were incubated with 1.5 % blocking serum in TRIS buffer solution at room temperature for up to 1 hour. The tissue samples were then incubated with the primary antibody at room temperature for 1 hour followed by flushing 3 times over 5 minutes with TRIS buffer solution. The tissue samples were then incubated with biotinized goat immunoglobulin (a biotin-containing secondary antibody) for 30 minutes and then flushed again 3 times over 5 minutes with TRIS buffer solution. An avidin and biotin-horseradish peroxidase complex was then later added, and tissue was incubated for 30 minutes at room temperature followed by flushing 3 times in 5 minutes with TRIS buffer solution. The tissue was then coated with the DAB Substrate Kit chromogenic system and incubated at room temperature for up to 10 minutes to obtain the brown colouration of the immunopositive structures.

Regardless of the type of staining system used, after incubation with the chromogenic substrate, the samples were flushed in running water and contrasted for two minutes with haematoxylin (code 05-M06002, Mayer's Hematoxylin, Bio

OPTICA Milano S.p.A., Italy). In conclusion, all prepared preparations were dehydrated with alcohol at increasing concentrations and clarified with xylol and carboxyxylol. The painted preparations were analysed with a light microscope (Leica DM500RB, Leica Biosystems Richmond Inc., United States of America) and treated with the Image-Pro Plus computer programme. Tissue sample microphotographs were captured with a digital camera (Leica DC 300F, Leica Microsystem AG, Germany).

Positive controls were prepared for all sample batches in tissues specified by antibody manufacturers where a positive reaction can always be detected. For the negative control, tissue sections were used in which the primary antibody was replaced by an antibody diluent.

1.3 Data processing methods

Review sections of all tissue preparations were evaluated according to the following criteria:

1) by morphological features of the surface epithelium (vacuolisation in epithelial cells, proliferation of the basal cell layer, hyperplasia, infiltration of intraepithelial inflammatory cells, degree of keratinization);
2) by the characterisation of subepithelial connective tissue cells and connective tissue fibres (fibrosis);
3) the presence or absence of inflammatory cell infiltration.

1.3.1 Semiquantitative counting method

The relative number of immunohistochemically positive structures was determined according to the semiquantitative counting method (Pilmane et al., 1998):

- 0 – no immunoreactive structures in the visual field;
- 0/+ – a rare occurrence of immunoreactive structures in the visual field;
- + – a few immunoreactive structures in the visual field;
- +/++ – few to moderate number of immunoreactive structures in the visual field;
- ++ – a moderate number of immunoreactive structures in the visual field;
- ++/+++ – moderate to numerous immunoreactive structures in the visual field;
- +++ – numerous immunoreactive structures in the visual field;
- +++/++++ – numerous to abundant number of immunoreactive structures in the visual field;
- ++++ – abundant number of immunoreactive structures in the visual field.

The relative number of reactive structures was evaluated in 5 randomly selected fields of vision per each section. The median number of structures in each of these fields of vision per slide was applied for further analysis.

1.3.2 Statistical methods

The data of the study were evaluated using descriptive statistical methods. The immunoreactivity of each cleft candidate gene protein in each group was described by calculating the median and interquartile range. Statistically

significant differences between several unrelated groups were determined using the Kruskal-Wallis H test (Chan and Walmsley, 1997). Statistically significant differences between the data of study groups and the control group were determined using the Mann-Whitney U test (Altman, 1990). The Spearman rank correlation coefficient (Forthofer et al., 2007) was used to evaluate the correlations between two variables. Calculated correlations were interpreted as follows: 0.0–0.2 was rated as a very weak correlation, 0.2–0.4 – a weak correlation, 0.4–0.6 – a moderately strong correlation, 0.6–0.8 – a strong correlation, 0.8–1.0 – a very strong correlation. The results were considered statistically significant at $p < 0.05$. In data statistics, the analysis was provided with Statistical product and Service solutions (SPSS) Statistics version 25.0 (IBM company, Chicago, Illinois, United States of America).

2 Results

2.1 Morphological findings

A histological finding consistent with normal oral mucosa tissue was established in the preparations obtained from seven patients of the first control group (patients who received plastic surgery of the upper lip frenulum plastic surgery). The preparations of the second control group (patients with oral tissue derived from the historical collection of RSU AAI) derived from upper lip tissue from five patients also showed a normal histological finding.

Oral epithelium and mucosal connective tissue were found in tissue samples of cleft lip tissue obtained during lip primary plastic surgery and cleft palate tissue obtained during soft palate plastic surgery. In all three groups of cleft patients, the routine histological finding was similar to relatively healthy oral mucosa tissue, but separate morphological variations were identified.

Some cleft lip tissue samples showed slight vacuolization of surface epithelial cells. No vacuolization of epithelial cells was observed in patients with cleft palate. Uneven proliferation of basal layer cells of the oral cavity epithelium was found in some cleft tissue specimens.

In some micropreparations, a minimal subepithelial inflammatory cell infiltrate was found, which was observed in some cleft patients. Some samples of cleft palate tissue, along with subepithelial inflammatory cell infiltrate, showed fibrotic changes in the connective tissue of the lamina propria.

2.2 Immunohistochemical characterization of cleft candidate genes

2.2.1 BARX1 gene protein

BARX1 gene protein was not detected (0) in the epithelium of the control group. In most control patients, no BARX1 positive cells (0) were found in the connective tissue, but in the connective tissue of one control patient, BARX1 positive cells had a rare occurrence (0/+).

BARX1 gene protein was not detected in the epithelium of the unilateral cleft lip group (0), but in the connective tissue overall, BARX1 positive cells had a rare occurrence (0/+) (see Figure 2.1).

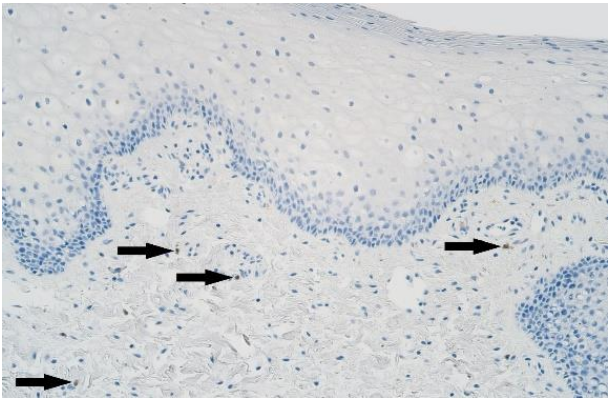

Figure 2.1 **A rare occurrence (0/+) of** BARX1 **positive connective tissue cells (arrows) in a three-month-old patient with a complete unilateral cleft lip and palate-lip plastic surgery**

BARX1 IMH, × 200.

BARX1 gene protein was not detected (0) in the epithelium of the bilateral cleft lip group, but overall in connective tissue, BARX1 positive cells had a rare occurrence (0/+).

BARX1 gene protein was not detected (0) in the epithelium of the cleft palate group, but in the connective tissue, BARX1 positive cells had a rare occurrence (0/+) in half of the samples, while the other half had no BARX1 positive cells (0).

Statistically significant differences in epithelium were not found between the groups of cleft patients and the control group in the immunoreactivity of the BARX1 gene protein when using the Kruskal-Wallis H test, but a statistically significant difference was determined in the connective tissue between all groups of cleft tissue and the control group ($H = 27.921$, $df = 3$, $p < 0.001$).

When comparing the groups with the Mann-Whitney U test, statistically significant differences in the immunoreactivity of the BARX1 gene protein were not detected in the epithelium, but statistically significant differences in connective tissue were calculated between the control group and the unilateral cleft lip group ($U = 17.50$, $p = 0.002$), between the unilateral cleft lip and the bilateral cleft lip group ($U = 149.50$, $p = < 0.001$).

2.2.2 DLX4 gene protein

DLX4 gene protein in the control group as a whole was detected in a few (+) cells in both epithelial and connective tissue.

DLX4 gene protein in the unilateral cleft lip group as a whole was detected in a few (+) cells in both epithelial and connective tissue.

DLX4 gene protein in the bilateral cleft lip group was generally found in a few (+) cells in the epithelium and in a rare occurrence (0/+) in connective tissue (see Figure 2.2).

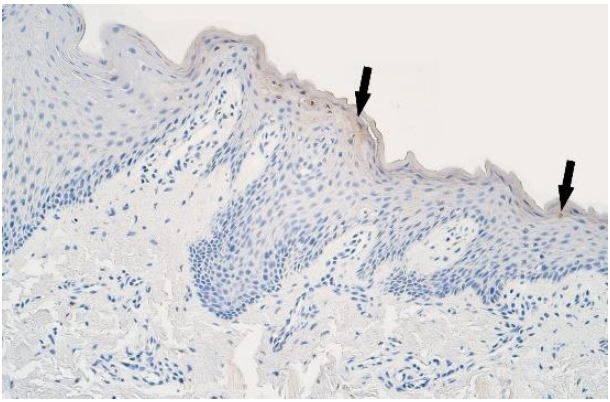

Figure 2.2 **Few (+)** DLX4 **positive epithelial cells and intraepithelial lymphocytes (arrows) in a seven-month-old patient with a complete bilateral cleft lip and palate-lip plastic surgery**

DLX4 IMH, × 200

DLX4 gene protein in the epithelium of the cleft palate group was generally rarely detected (0/+) and had a rare occurrence to a few (0/+ to +) positive connective tissue cells.

Statistically significant differences were found in both epithelial tissue (H = 10,541, df = 3, p = 0.014) **and connective tissue** (H = 9,395, df = 3, p = 0.024) between all cleft patient groups and the control group.

In a group-to-group comparison with the Mann-Whitney U test, statistically significant differences in the immunoreactivity of the DLX4 gene protein were determined in the epithelium between the unilateral cleft lip group and the cleft palate group (U = 264.50, p = 0.003) and between the bilateral cleft lip group and the cleft palate group (U = 99.00, p = 0.037). Conversely, the statistically significant differences in connective tissue were determined between the control group and the bilateral cleft lip group (U = 16.50, p = 0.019) and between the control group and the cleft palate group (U = 39.50, p = 0.021).

2.2.3 FOXE1 gene protein

FOXE1 gene protein in the control group epithelium was generally found in few to moderate (+/++) number and in a few (+) cells in connective tissue.

FOXE1 gene protein in the unilateral cleft lip group in general was determined in moderate number (++) in both epithelial and connective tissue (see Figure 2.3).

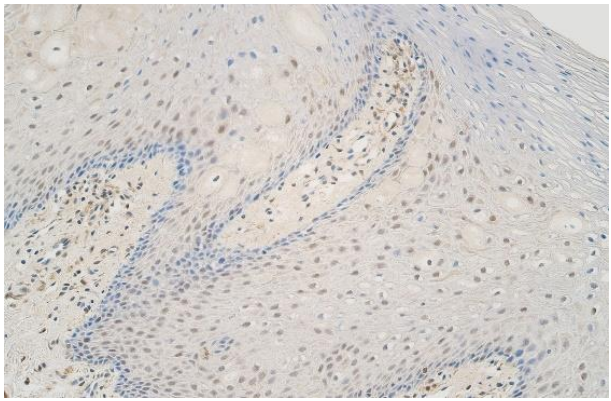

Figure 2.3 **Moderate to numerous (++/+++) FOXE1-containing epithelial and connective tissue cells in a seven-month-old patient with a complete unilateral cleft lip and palate-lip plastic surgery**

FOXE1 IMH, × 200.

FOXE1 gene protein in was generally detected in the epithelium the bilateral cleft lip group in a few to moderate (+/++) number and in connective tissue – in a few (+) cells.

FOXE1 gene protein in the cleft palate group was generally found in a few (+) cells in both epithelial and connective tissue (see Figure 2.4).

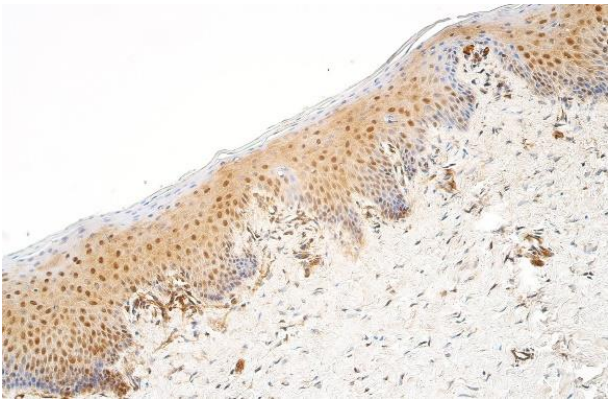

Figure 2.4 **Numerous (+++) FOXE1 positive epithelial cells and moderate number (++) of subepithelial connective tissue cells in a four-month-old patient with a complete unilateral cleft lip and palate – soft palate plastic surgery**

FOXE1 IMH, × 200.

No statistically significant difference in the immunoreactivity of the FOXE1 gene protein was detected between cleft patient groups and the control group in the epithelium, but **a statistically significant difference was observed in connective tissue** ($H = 13.713$, $df = 3$, $p = 0.003$).

When comparing the groups with the Mann-Whitney U test, a statistically significant difference in the immunoreactivity of the FOXE1 gene protein was established in the connective tissue between control patients and unilateral cleft lip patients ($U = 28.00$, $p = 0.011$), between unilateral cleft lip patients and bilateral cleft lip patients ($U = 148.50$, $p = 0.050$), and between unilateral cleft lip patients and cleft palate patients ($U = 252.50$, $p = 0.002$).

2.2.4 HOXB3 gene protein

HOXB3 gene protein in the control group was generally found in a few (+) epithelial cells and in a moderate number (+ +) of cells in connective tissue.

HOXB3 gene protein in the unilateral cleft lip group was generally determined in moderate to numerous (++/+++) epithelial cells and in moderate number (++) in the connective tissue (see Figure 2.5).

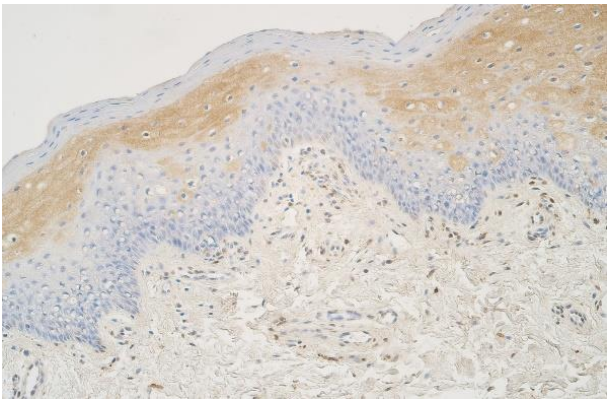

Figure 2.5 **Moderate number (++) of HOXB3 positive epithelial and connective tissue cells in a seven-month-old patient with a complete unilateral cleft lip and palate-lip plastic surgery**

HOXB3 IMH, × 200

HOXB3 gene protein in the bilateral cleft lip group was generally found in a moderate (++) number in the epithelium and in few to moderate (+/++) number of cells in the connective tissue (see Figure 2.6).

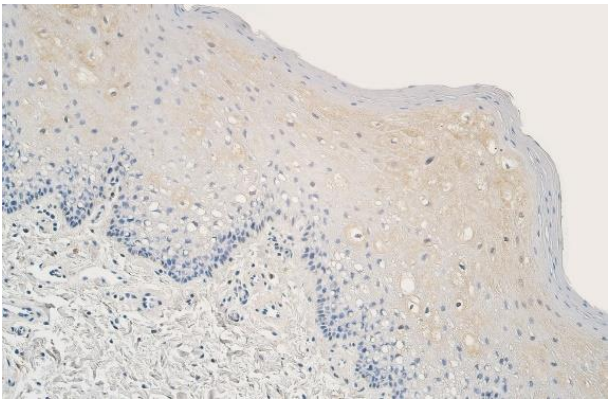

Figure 2.6 **Moderate to numerous (++/+++) lightly stained HOXB3 positive epithelial cells in a six-month-old patient with a complete bilateral cleft lip and palate-lip plastic surgery**

HOXB3 IMH, × 200

HOXB3 gene protein in the cleft palate group was generally found in few to moderate – moderate (+/++ to ++) number in the epithelium and in few to moderate (+/++) number in the connective tissue.

When assessing differences in immunoreactivity of the HOXB3 gene protein between the groups using the Kruskal-Wallis H test, **statistically significant differences were identified in both epithelial** ($H = 20.137$, $df = 3$, $p < 0.001$) **and connective tissue** ($H = 8.419$, $df = 3$, $p < 0.038$) between cleft patient groups and the control group.

When comparing the groups with the Mann-Whitney U test, statistically significant differences in the immunoreactivity of the HOXB3 gene protein were found in the epithelium between the control group and the unilateral cleft lip group ($U = 23.00$, $p = 0.005$), between the unilateral cleft lip group and the bilateral cleft lip group ($U = 113.00$, $p = 0.005$), between the unilateral cleft lip group and the cleft palate group ($U = 212.50$, $p < 0.001$). Statistically significant differences in connective tissue in the immunoreactivity of the HOXB3 gene protein were determined between the unilateral cleft lip group and the bilateral

cleft lip group (U = 127,00, p = 0,011), between the unilateral cleft lip group and the cleft palate group (U = 324,00, p = 0,035).

2.2.5 MSX2 gene protein

MSX2 gene protein was generally not detected (0) in the epithelium of control group in any samples, but in connective tissue MSX2 was not detected (0) in three cases but had a rare occurrence (0/+) in two cases.

MSX2 gene in the epithelium of the unilateral cleft lip group was generally determined in moderate number (++) of cells and in a few (+) cells in connective tissue (see Figure 2.7).

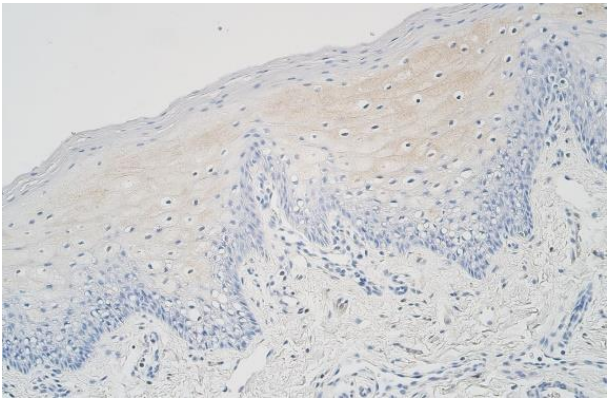

Figure 2.7 **Moderate (++) number of weakly positive MSX2-containing epithelial cells in a seven-month-old patient with a complete unilateral cleft lip and palate-lip plastic surgery**

MSX2 IMH, × 200

MSX2 gene protein in the bilateral cleft lip group was generally found in a few (+) cells in the epithelium and in a rare occurrence (0/+) in connective tissue.

MSX2 gene protein in the cleft palate group had a rare occurrence (0/+) as a whole both in epithelial and connective tissue (see Figure 2.8).

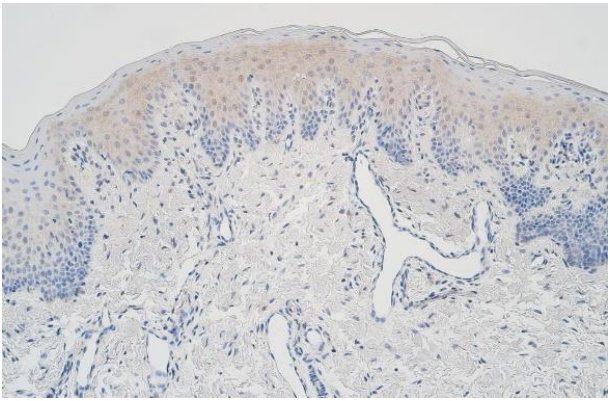

Figure 2.8. Moderate (++) number of MSX2 positive epithelial cells in an eight-month-old patient with a complete unilateral cleft lip and palate – soft palate plastic surgery

MSX2 IMH, × 200

A statistically significant difference in both epithelial ($H = 32{,}722$, $df = 3$, $p < 0.001$) **and connective tissue** ($H = 15{,}167$, $df = 3$, $p = 3$, $p = 0.002$) between cleft patient groups and the control group was determined by evaluating the differences in immunoreactivity of the MSX2 gene protein between the groups with the Kruskal-Wallis H test.

In a group-to-group comparison, statistically significant differences in immunoreactivity of the MSX2 gene protein in the epithelium were observed between the control group and the unilateral cleft lip group ($U = 7.50$, $p < 0.001$), between the control group and the bilateral cleft lip group ($U = 10.00$, $p = 0.026$), between the control group and the cleft palate group ($U = 27.50$, $p = 0.041$), between the unilateral cleft lip group and the bilateral cleft lip group ($U = 107.00$, $p = 0.003$), between the unilateral cleft lip group and the cleft palate group ($U = 134{,}50$, $p < 0{,}001$). Statistically significant differences in the

immunoreactivity of the MSX2 gene protein gene were determined between the control group and the unilateral cleft lip group (U = 25.00, p = 0.007), between the unilateral cleft lip group and the bilateral cleft lip group (U = 124.00, p = 0.010) and between the unilateral cleft lip group and the cleft palate group (U = 274.00, p = 0.008).

2.2.6 PAX7 gene protein

PAX7 gene protein in the control group was generally found in few to moderate (+/++) number of cells in both in epithelial and connective tissue.

PAX7 gene protein in the epithelium of the unilateral cleft lip group was generally found in moderate to numerous (++/+++) cells and in connective tissue – in numerous (+++) cells (see Figure 2.9).

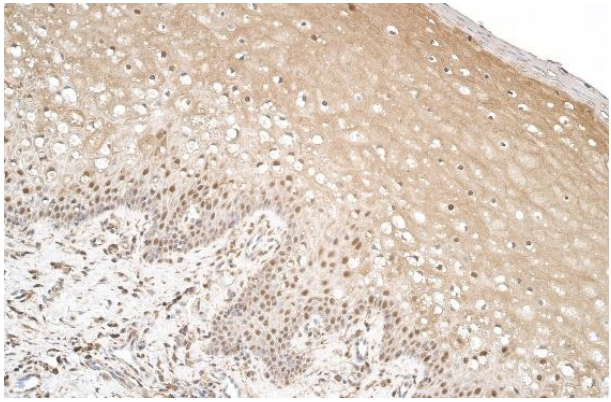

Figure 2.9 **Numerous to abundant (+++/++++) PAX7 positive epithelial cells and moderate (++) number of connective tissue cells in a four-month-old patient with a complete unilateral cleft lip and palate – lip plastic surgery**

PAX7 IMH, × 200

PAX7 gene protein in the bilateral cleft lip group was generally found in a moderate (++) number in the epithelium and in a few to moderate (+/++) number in connective tissue (see Figure 2.10).

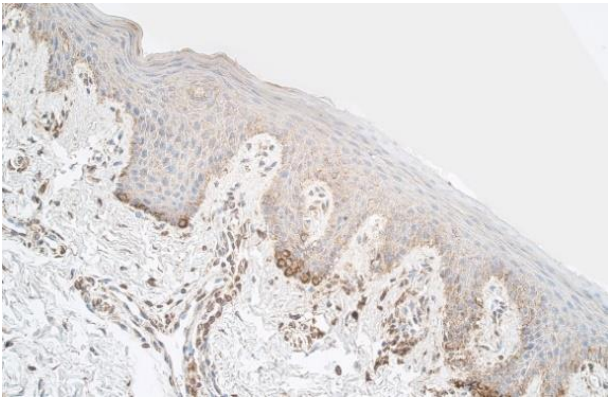

Figure 2.10 **Few to moderate (+/++) number of PAX7 positive epithelial and connective tissue cells in a four-month-old patient with a complete bilateral cleft lip and palate – lip plastic surgery**

PAX7 IMH, × 200

PAX7 gene protein in the cleft palate group epithelium was generally detected in a few to moderate (+/++) number of cells and in connective tissue – in moderate to numerous (++/+++) number of cells.

A statistically significant difference was identified in both epithelial ($H = 31.195$, $df = 3$, $p < 0.001$) **and connective tissue** ($H = 29.976$, $df = 3$, $p < 0.001$) between cleft patient groups and the control group when evaluating the differences in immunoreactivity of the PAX7 gene protein between groups with the Kruskal-Wallis H test.

When comparing the groups with the Mann-Whitney U test, statistically significant differences in the immunoreactivity of the PAX7 gene protein in the epithelium were found between the control group and the unilateral cleft lip group ($U = 6.50$, $p < 0.001$), between the unilateral cleft lip and the bilateral cleft

lip group (U = 82.00, p < 0.001), between the unilateral cleft lip and the cleft palate group (U = 148.50, p < 0.001). Statistically significant differences in the immunoreactivity of the PAX7 gene protein were determined between the control group and the unilateral cleft lip group (U = 14.50, p = 0.001), between the unilateral cleft lip group and the bilateral cleft lip group (U = 40.50, p < 0.001), between the unilateral cleft lip group and the cleft palate group (U = 226.00, p < 0.001), and between the bilateral cleft lip group and the cleft palate group (U = 101.50, p = 0.043).

2.2.7 PAX9 gene protein

PAX9 gene protein in the control group was generally found in a moderate number (++) in the epithelium, while in connective tissue it was not detected (0) in four cases and had a rare occurrence (0/+) in three cases.

PAX9 gene protein in the unilateral cleft lip group was generally found in a few to moderate (+/++) number of cells in the epithelium and in a moderate (++) number in connective tissue (see Figure 2.11).

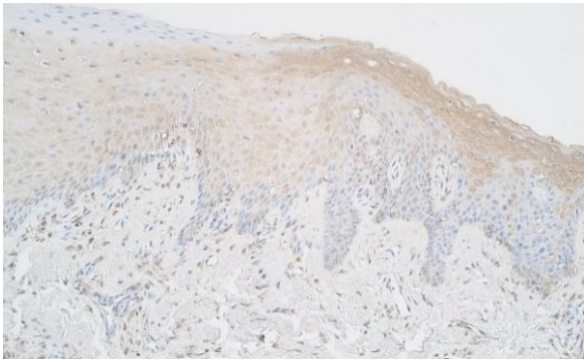

Figure 2.11 **Moderate to numerous (++/+++) PAX9 positive epithelial and connective tissue cells in a four-month-old patient with a complete unilateral cleft lip and palate – lip plastic surgery**

PAX9 IMH, × 200

PAX9 gene protein in the bilateral cleft lip tissue had a rare occurrence (0/+) as a whole in both epithelial and connective tissue.

PAX9 gene protein in the cleft palate tissue generally had a rare occurrence (0/+) in the epithelium and had a few (+) positive cells in the connective tissue (see Figure 2.12).

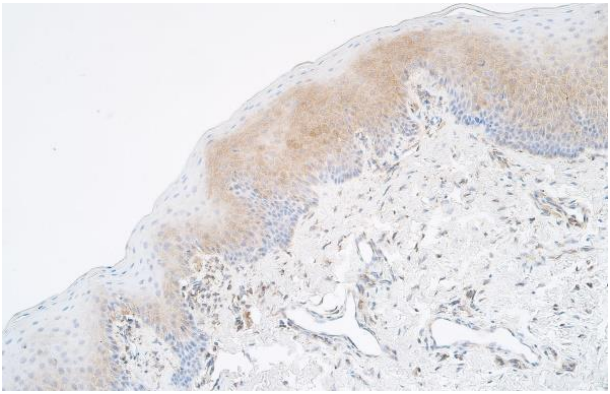

Figure 2.12 **Moderate to numerous (++/+++) PAX9 positive epithelial and connective tissue cells in an eight-month-old patient with a complete unilateral cleft lip and palate – soft palate plastic surgery**

PAX9 IMH, × 200

When evaluating differences in immunoreactivity of the PAX9 gene protein, **statistically significant differences were identified in both the epithelium** ($H = 32.333$, $df = 3$, $p < 0.001$) **and connective tissue** ($H = 38.245$, $df = 3$, $p < 0.001$) between cleft patient groups and the control group.

In a group-to-group comparison, statistically significant differences in PAX9 gene protein immunoreactivity were found in the epithelium between the control group and the bilateral cleft lip group ($U = 5.00$, $p < 0.001$), between the control group and the cleft palate group ($U = 2.50$, $p = 0.003$), between the unilateral cleft lip group and the cleft palate group ($U = 145.00$, $p < 0.001$). In connective tissue, statistically significant differences in PAX9 gene protein

immunoreactivity were determined between the control group and the unilateral cleft lip group ($U = 9.50$, $p < 0.001$), between the control group and the cleft palate group ($U = 18.50$, $p = 0.001$), between the unilateral cleft lip group and the bilateral cleft lip group ($U = 48.00$, $p < 0.001$), between the unilateral cleft lip group and the cleft palate group ($U = 188.50$, $p < 0.001$), and between the bilateral cleft lip group and the cleft palate group ($U = 81.00$, $p = 0.008$).

2.2.8 RYK gene protein

RYK gene protein in the control group as a whole had a rare occurrence (0/+) in both epithelial and connective tissue.

RYK gene protein in the unilateral cleft lip group was determined in moderate to numerous (++/+++) cells in both epithelial and connective tissue in general (see Figure 2.13).

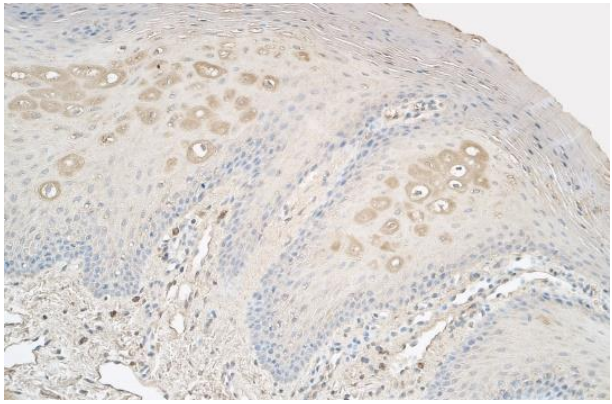

Figure 2.13 **Moderate number (++) of RYK positive epithelial cells and moderate (++) of RYK positive connective tissue cells in an eight-month-old patient with a complete unilateral cleft lip and palate – lip plastic surgery**

RYK IMH, × 200

RYK gene protein in the bilateral cleft lip group was found on average in a moderate to numerous (++/+++) number of cells in both epithelial and connective tissue (see Figure 2.14).

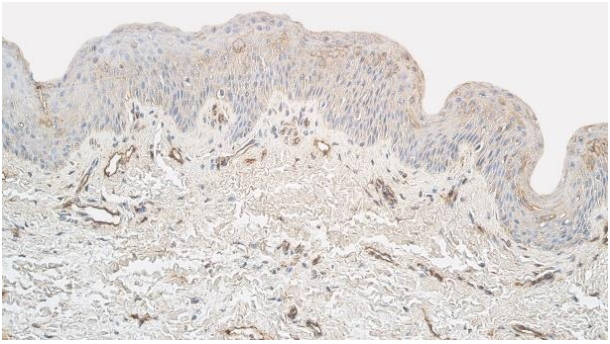

Figure 2.14 **Moderate (++) number of RYK positive connective tissue cells and numerous (+++) endothelial cells in a seven-month-old patient with a complete bilateral cleft lip and palate – lip plastic surgery**

RYK IMH, × 200

RYK gene protein in the cleft palate tissue group was found in a moderate to numerous (++/+++) number of cells both in epithelial and connective tissue.

A statistically significant difference was identified in both the epithelial ($H = 22.932$, $df = 3$, $p < 0.001$) **and connective tissue** ($H = 18.443$, $df = 3$, $p < 0.001$) between cleft patient groups and the control group when assessing the differences in immunoreactivity of RYK gene protein between groups.

In a group-to-group comparison, statistically significant differences in RYK gene protein immunoreactivity were found in the epithelium between the control group and the unilateral cleft lip group ($U = 5.50$, $p < 0.001$), between the control group and the bilateral cleft lip group ($U = 3.50$, $p < 0.001$), between the control group and the cleft palate group ($U = 9.50$, $p < 0.001$), between the

unilateral cleft lip group and the cleft palate group (U = 321.50, p = 0.030). Statistically significant differences in the immunoreactivity of the RYK gene protein were determined between the control group and the unilateral cleft lip group (U = 11.00, p < 0.001), between the control group and the bilateral cleft lip group (U = 2.50, p < 0.001) and between the control group and the cleft palate group (U = 5.00, p < 0.001).

2.2.9 SHH gene protein

SHH gene protein in the control group was generally found in moderate to numerous (++/+++) number in the epithelium and in moderate (++) number in connective tissue (++).

The SHH gene protein in unilateral cleft lip group was found in a moderate to numerous (++/+++) number in both epithelial and connective tissue (see Figure 2.15).

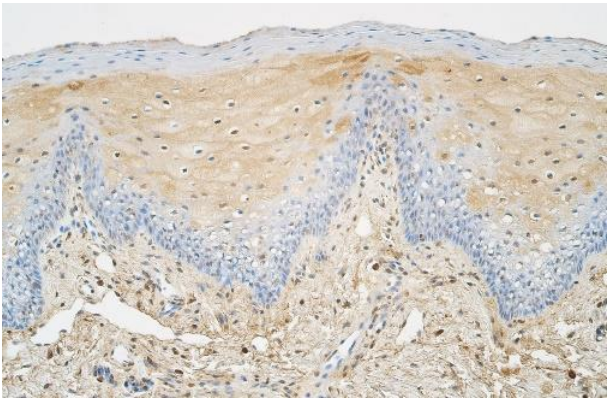

Figure 2.15 **Moderate to numerous (++/+++) SHH positive epithelial and connective tissue cells in a seven-month-old patient with a complete unilateral cleft lip and palate – lip plastic surgery**

SHH IMH, × 200

SHH gene protein in the bilateral cleft lip group was generally detected in a few to moderate (+/++) number of cells in both epithelial and connective tissue. (see Figure 2.16).

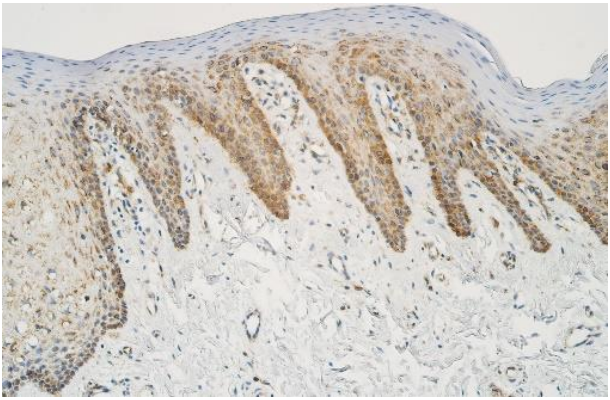

Figure 2.16 **Moderate to numerous (++/+++) SHH positive epithelial cells in a six-month-old patient with a complete bilateral cleft lip and palate – lip plastic surgery**

SHH IMH, × 200

SHH gene protein in the cleft palate group was generally found in a few (+) epithelial cells and in a few to moderate-moderate (+/++ to ++) number of connective tissue cells.

S**tatistically significant differences were identified in both epithelium** ($H = 47.119$, $df = 3$, $p < 0.001$) **and connective tissue** ($H = 41.192$, $df = 3$, $p < 0.001$) between the cleft patient groups and the control group.

In a group-to-group comparison, statistically significant differences in the immunoreactivity of the SHH gene protein were found in the epithelium between the control group and the bilateral cleft lip group ($U = 9.00$, $p = 0.019$), between the control group and the cleft palate group ($U = 4.00$, $p < 0.001$), between the unilateral cleft lip group and the cleft palate group ($U = 37.00$, $p < 0.001$), between the bilateral cleft lip group and the cleft palate group ($U = 94.00$,

p = 0.025). Statistically significant differences in the immunoreactivity of the SHH gene protein were determined between the control group and the cleft palate group (U = 14.00, p = 0.004), between the unilateral cleft lip group and the bilateral cleft lip group (U = 72.50, p < 0.001) and between the unilateral cleft lip group and the cleft palate group (U = 62.00, p < 0.001).

2.2.10 SOX3 gene protein

SOX3 gene protein in the control group was generally found in numerous to abundant (+++/++++) number in the epithelium and in numerous (+++) connective tissue cells.

SOX3 gene protein in the unilateral cleft lip patients was generally found in a moderate to numerous (++/+++) number of epithelial cells and in a moderate (++) number of connective tissue cells (see Figure 2.17).

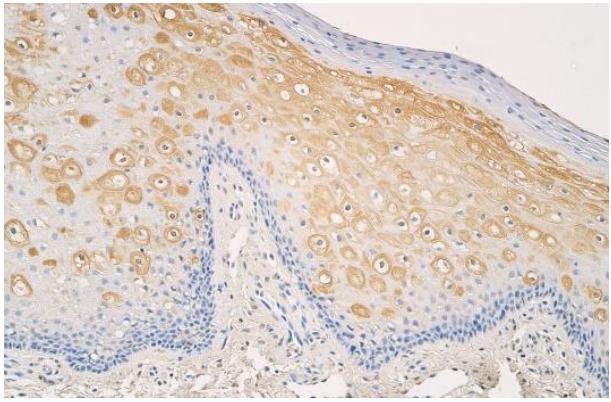

Figure 2.17 **Numerous (+++) SOX3 positive epithelial cells in a three-month-old patient with a complete unilateral cleft lip and palate – lip plastic surgery**

SOX3 IMH, × 200

SOX3 gene protein in the bilateral cleft lip group was generally found in a moderate (++) number in both epithelial and connective tissue (see Figure 2.18).

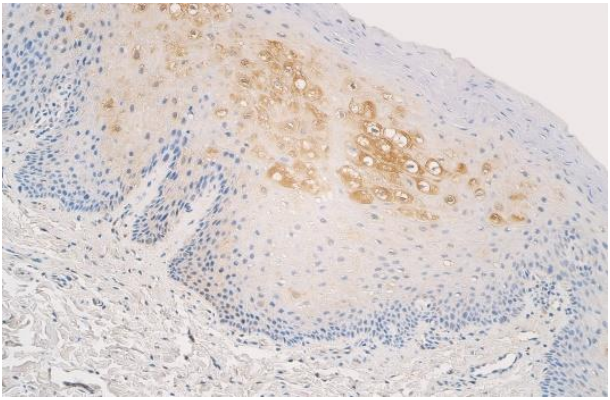

Figure 2.18 **Moderate (++) number of SOX3 positive epithelial cells in a six-month-old patient with a complete bilateral cleft lip and palate – lip plastic surgery**

SOX3 IMH, × 200

SOX3 gene protein in the cleft palate group was found in few to moderate (+/++) epithelial cells and in a moderate (++) number of connective tissue cells.

When assessing the differences in SOX3 gene protein immunoreactivity between groups, **statistically significant differences were identified in both the epithelium** ($H = 34.856$, $df = 3$, $p = 0.005$) **and connective tissue** ($H = 12.838$, $df = 3$, $p < 0.001$) between cleft patient groups and the control group.

In a group-to-group comparison, statistically significant differences in the immunoreactivity of the SOX3 gene protein were observed in the epithelium between the control group and the unilateral cleft lip group ($U = 14.50$, $p = 0.001$), between the control group and the bilateral cleft lip group ($U = 9.00$,

p = 0.019), between the control group and the cleft palate group (U = 1.00, p < 0.001), between the unilateral cleft lip group and the bilateral cleft lip group (U = 121.50, p = 0.008), between the unilateral cleft lip group and the cleft palate group (U = 140.00, p < 0.001). Statistically significant differences in the immunoreactivity of the SOX3 gene protein were determined between the control group and the unilateral cleft lip group (U = 10.500, p < 0.001), between the control group and the bilateral cleft lip group (U = 4.50, p = 0.003), between the control group and the cleft palate group (U = 4.00, p < 0.001).

2.2.11 WNT3A gene protein

WNT3A in control group patients was observed in a moderate to numerous (++/+++) number of epithelial cells and in numerous (+++) connective tissue cells.

WNT3A in unilateral cleft lip patients was detected in a few (+) epithelial and connective tissue cells (see Figure 2.19).

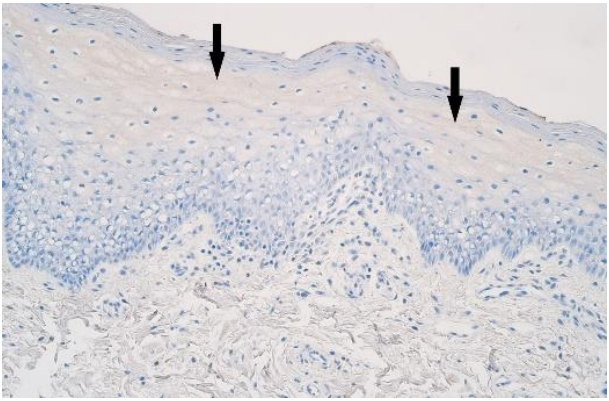

Figure 2.19 **Few to moderate (+/++) number of very weakly positive WNT3A-containing epithelial cells (arrows) in a seven-month-old patient with a complete unilateral cleft lip and palate – lip plastic surgery**

WNT3A IMH, × 200

WNT3A in bilateral cleft lip patients had a rare occurrence (0/+) in the epithelium and a few (+) positive connective tissue cells.

WNT3A in cleft palate patients had a rare occurrence (0/+) in both the epithelium and the connective tissue.

When assessing the differences in the immunoreactivity of WNT3A gene protein between the groups, **statistically significant differences were identified in both the epithelium** ($H = 24.529$, $df = 3$, $p < 0.001$) **and connective tissue** ($H = 19.326$, $df = 3$, $p < 0.001$) between the cleft patient groups and the control group.

When comparing the groups with the Mann-Whitney U test, statistically significant differences were found in the immunoreactivity of the WNT3A gene protein in the epithelium between the control group and the unilateral cleft lip group ($U = 19.00$, $p = 0.002$), between the control group and the bilateral cleft lip group ($U = 1.00$, $p < 0.001$), between the control group and the cleft palate group ($U = 113.00$, $p = 0.005$), between the unilateral cleft lip group and the cleft palate group ($U = 247.50$, $p = 0.001$). Statistically significant differences in connective tissue were determined in immunoreactivity of the WNT3A gene protein between the control group and the unilateral cleft lip group ($U = 0.00$, $p < 0.001$), between the control group and the bilateral cleft lip group ($U = 0.00$, $p = 0.003$), between the control group and the cleft palate group ($U = 0.00$, $p < 0.001$).

2.2.12 WNT9B gene protein

WNT9B in control patients was determined in numerous to abundant (+++/++++) number of epithelial and connective tissue cells.

WNT9B in unilateral cleft lip patients was generally found to be in moderate to numerous (++/+++) epithelial cells and in numerous (+++) connective tissue cells (see Figure 2.20).

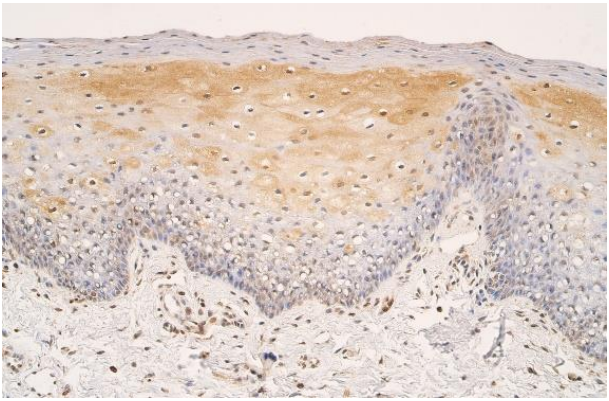

Figure 2.20 **Moderate to numerous (++/+++) WNT9B positive epithelial and connective tissue cells in a seven-month-old patient with a complete unilateral cleft lip and palate – lip plastic surgery**

WNT9B IMH, × 200

WNT9B in bilateral cleft lip patients on average had a moderate (++) number of epithelial cells and moderate to numerous (++/+++) connective tissue cells.

WNT9B in cleft palate patients a moderate (++) number of epithelial cells and moderate to numerous (++/+++) connective tissue cells.

When assessing the differences in the immunoreactivity of the WNT9B gene protein between the groups, **statistically significant differences were identified in both the epithelium** ($H = 26.976$, $df = 3$, $p = 0.005$) **and connective tissue** ($H = 20.123$, $df = 3$, $p < 0.001$) between the cleft patient groups and the control group.

In a group-to-group comparison, statistically significant differences were observed in the immunoreactivity of the WNT9B gene protein in the epithelium between the control group and the unilateral cleft lip group ($U = 14.00$, $p = 0.001$), between the control group and the bilateral cleft lip group ($U = 0.50$, $p < 0.001$), between the control group and the cleft palate group ($U = 1.00$, $p < 0.001$), between the unilateral cleft lip group and the bilateral cleft lip group

(U = 143.50, p = 0.033), between the unilateral cleft lip group and the cleft palate group (U = 204.00, p < 0.001). Statistically significant differences in connective tissue were determined in immunoreactivity of the WNT9B gene protein between the control group and the unilateral cleft lip group (U = 32.50, p = 0.018), between the control group and the bilateral cleft lip group (U = 4.50, p = 0.003), between the control group and the cleft palate group (U = 2.50, p < 0.001) and between the unilateral cleft lip group and the cleft palate group (U = 246.50, p = 0.001).

2.3 Data about correlations between immunohistochemically determined cleft candidate genes

A statistically significant strong positive correlation was observed between the age of the patients and the relative number of RYK positive connective tissue cells (r_s = 0.768, p = 0.044) was observed in the first control group (patients who had plastic surgery of the upper lip frenulum plastic surgery). In the first control group, **several statistically significant very strong correlations were identified** between cleft candidate gene proteins. Positive correlations primarily involved DLX4 in epithelium and in connective tissue, which were found in four statistically significant positive correlations. DLX4 was also found in two statistically significant negative correlations. In statistically significant positive correlations, PAX7 involvement was observed in three cases.

A **statistically significant strong correlation was identified** between SHH in the epithelium and SHH in connective tissue (r_s = 0.892, p = 0.042) was identified in the second control group (patients whose oral tissue was obtained from the historical collection of RSU AAI).

In the unilateral cleft lip group, one hundred and four statistically significant strong, moderate, and weak correlations were identified between the evaluated cleft candidate genes. The **strongest statistically significant correlations** in the unilateral cleft lip group are summarised in Table 2.1.

Table 2.1

Statistically significant strong and moderate positive correlations ($p \leq 0.001$) of immunohistochemically determined cleft candidate gene proteins in the tissue of unilateral cleft lip

Gene proteins between which correlation has been established	Spearman correlation coefficient	p value
MSX2 in epithelium and MSX2 in connective tissue	0.710	< 0.001
HOXB3 in epithelium and HOXB3 in connective tissue	0.684	< 0.001
HOXB3 in epithelium and PAX9 in epithelium	0.663	< 0.001
WNT3A in epithelium and WNT9B in epithelium	0.662	< 0.001
WNT9B in epithelium and WNT9B in connective tissue	0.662	< 0.001
DLX4 in epithelium and HOXB3 in epithelium	0.632	< 0.001
PAX9 in epithelium and PAX9 in connective tissue	0.632	< 0.001
RYK in epithelium and RYK in connective tissue	0.604	< 0.001
SOX3 in epithelium and SOX3 in connective tissue	0.599	< 0.001
FOXE1 in connective tissue and PAX9 in connective tissue	0.596	< 0.001
WNT3A in epithelium and WNT3A in connective tissue	0.594	< 0.001
SHH in epithelium and WNT9B in epithelium	0.578	< 0.001
HOXB3 in epithelium and WNT9B in connective tissue	0.576	< 0.001
FOXE1 in epithelium and FOXE1 in connective tissue	0.561	< 0.001
PAX7 in connective tissue and WNT9B in connective tissue	0.561	< 0.001
FOXE1 in connective tissue and HOXB3 in connective tissue	0.552	< 0.001
HOXB3 in connective tissue and SHH in connective tissue	0.546	0.001
HOXB3 in connective tissue and PAX9 in connective tissue	0.543	0.001
WNT3A in connective tissue and WNT9B in epithelium	0.542	0.001
MSX2 in epithelium and WNT3A in epithelium	0.541	0.001
PAX7 in epithelium and PAX7 in connective tissue	0.541	0.001
FOXE1 in connective tissue and SOX3 in connective tissue	0.538	0.001
PAX9 in connective tissue and SHH in connective tissue	0.532	0.001
PAX7 in connective tissue and SHH in connective tissue	0.530	0.001
HOXB3 in connective tissue and WNT9B in connective tissue	0.524	0.001
PAX7 in connective tissue and PAX9 in connective tissue	0.524	0.001

Table 2.1 continued

Gene proteins between which correlation has been established	Spearman correlation coefficient	p value
SOX3 in epithelium and WNT9B in epithelium	0.524	0.001
FOXE1 in connective tissue and PAX7 in connective tissue	0.514	0.001
HOXB3 in epithelium and WNT9B in epithelium	0.513	0.001
SHH in epithelium and WNT3A in epithelium	0.512	0.001

Legend: DLX4 – distal-less hoemeobox-4, FOXE1 – forkhead box E1, HOXB3 – homeobox B3, MSX2 – muscle segment homeobox-2, PAX7 – paired box transcription factor-7, PAX9 – paired box transcription factor-9, RYK – receptor-like tyrosine kinase, SHH – sonic hedgehog, SOX3 – SRY-box transcription factor-3, WNT3A – wingless-type MMTV integration site protein-3A, WNT9B – wingless-type MMTV integration site protein-9B.

A **statistically significant strong negative correlation was identified between the age of the patients and the relative amount of SOX3 in the connective tissue** ($r_s = -0.711$, $p = 0.006$) in the bilateral cleft lip group. Seventy-two statistically significant very strong, strong, and moderate positive correlations between the evaluated cleft candidate genes were identified in the bilateral cleft lip group. The **strongest statistically significant correlations** in the bilateral cleft lip group are summarised in Table 2.2.

Table 2.2

Statistically significant very strong and strong positive correlations ($p \leq 0.001$) of immunohistochemically determined cleft candidate gene proteins in the tissue of bilateral cleft lip

Gene proteins between which correlation has been established	Spearman correlation coefficient	p value
HOXB3 in epithelium and WNT3A in epithelium	0.940	< 0.001
PAX9 in epithelium and WNT9B in epithelium	0.909	< 0.001
FOXE1 in epithelium and FOXE1 in connective tissue	0.859	< 0.001
HOXB3 in epithelium and SHH in connective tissue	0.836	< 0.001

Table 2.2 continued

Gene proteins between which correlation has been established	Spearman correlation coefficient	p value
SHH in epithelium and WNT9B in epithelium	0.811	0.001
PAX9 in epithelium and SHH in epithelium	0.802	0.001
PAX9 in epithelium and PAX9 in connective tissue	0.788	0.001
MSX2 in epithelium and SOX3 in epithelium	0.786	0.001

Legend: FOXE1 – forkhead box E1, HOXB3 – homeobox B3, MSX2 – muscle segment homeobox-2, PAX7 – paired box transcription factor-7, PAX9 – paired box transcription factor-9, RYK – receptor-like tyrosine kinase, SHH – sonic hedgehog, SOX3 – SRY-box transcription factor-3, WNT3A – wingless-type MMTV integration site protein-3A, WNT9B – wingless-type MMTV integration site protein-9B.

Seventy-one statistically significant strong, moderate, and weak correlation between evaluated cleft candidate genes was identified in the cleft palate group. The **strongest statistically significant correlations** in the cleft palate group are summarised in Table 2.3.

Table 2.3

Statistically significant very strong, strong and moderate positive correlations ($p \leq 0.001$) of immunohistochemically determined cleft candidate gene proteins in the tissue of cleft palate

Gene proteins between which correlation has been established	Spearman correlation coefficient	p value
SOX3 in epithelium and WNT9B in epithelium	0.862	< 0.001
DLX4 in epithelium and SOX3 in epithelium	0.751	< 0.001
DLX4 in epithelium and WNT9B in epithelium	0.725	< 0.001
FOXE1 in connective tissue and SOX3 in connective tissue	0.686	< 0.001
PAX9 in connective tissue and SHH in connective tissue	0.668	< 0.001
PAX7 in epithelium and PAX7 in connective tissue	0.657	< 0.001
PAX9 in epithelium and PAX9 in connective tissue	0.653	< 0.001
HOXB3 in epithelium and PAX9 in epithelium	0.641	< 0.001
PAX7 in connective tissue and PAX9 in connective tissue	0.624	0.001
RYK in epithelium and SHH in epithelium	0.622	0.001
HOXB3 in epithelium and PAX9 in connective tissue	0.614	0.001

Table 2.3 continued

Gene proteins between which correlation has been established	Spearman correlation coefficient	p value
HOXB3 in epithelium and PAX7 in epithelium	0.611	0.001
PAX7 in epithelium and RYK in epithelium	0.608	0.001
PAX7 in connective tissue and WNT3A in connective tissue	0.606	0.001
PAX7 in epithelium and SOX3 in epithelium	0.599	0.001

Legend: DLX4 – distal-less hoemeobox-4, FOXE1 – forkhead box E1, HOXB3 – homeobox B3, MSX2 – muscle segment homeobox-2, PAX7 – paired box transcription factor-7, PAX9 – paired box transcription factor-9, RYK – receptor-like tyrosine kinase, SHH – sonic hedgehog, SOX3 – SRY-box transcription factor-3, WNT3A – wingless-type MMTV integration site protein-3A, WNT9B – wingless-type MMTV integration site protein-9B.

3 Discussion

Globally, most cleft candidate gene studies have been done in animals, but relatively few studies have been conducted with human tissue. Morphopathogenesis of nonsyndromic orofacial clefts is relatively uncertain because it is not possible to clearly identify just one particular gene or morphopathogenetic mechanism, which could be the most significant in the morphogenesis of facial cleft tissue.

In our study, we have detected the relative number of immunopositive cells for BARX1, DLX4, FOXE1, HOXB3, MSX2, PAX7, PAX9, RYK, SHH, SOX3, WNT3A and WNT9B gene proteins in the tissue of unilateral cleft lip, bilateral cleft lip, and cleft palate collected from children before and during the age of primary dentition.

In tissue sections, epithelial cell vacuolization was observed in some patients with unilateral cleft lip (in four cases) and bilateral cleft lip (in one case), but it was not observed in patients whose oral tissue was acquired during soft palate plastic surgery. Cytoplasmic vacuolization of oral epithelial cells has been previously described in studies evaluating the effects of other abnormal conditions on the oral epithelium, such as infections (Kerga et al., 2018; Temple et al., 2014), diabetes mellitus (Seifi et al., 2014; Jajarm et al., 2008), or postmortem changes in oral tissue (Patro et al., 2021). Interestingly, another study of cleft palate tissue also described oral epithelial cell vacuolization (Tellerman et al., 2019), which in turn was not observed in our study. Most likely, this morphological feature could be a nonspecific characteristic of oral epithelial cell damage and degeneration, which could be associated with the presence of a cleft.

In some cleft tissue specimens, epithelial basal cell proliferation was observed. This morphological characteristic is described in basal cell carcinoma (Florescu et al., 2018), leukoplakia, and oral squamous cell carcinoma (Thomson

et al., 2002), but has also been detected in facial cleft tissue (Tellerman et al., 2019). The morphopathogenesis of facial clefts is associated with changes in cell proliferation, but this morphological feature was described in the epithelium in only a few cleft tissue samples, suggesting that changes in cell proliferation are likely to have occurred prenatally in most patients and that epithelial basal cell proliferation activity is postnatally similar to normal oral tissue. The existing changes in these specific samples could be interpreted as a local morphological variation that could be associated with a cleft or some other morphopathogenetic process, such as inflammation, which is common in cleft-affected tissue.

In separate tissue overview sections, a minimum subepithelial inflammatory cell infiltrate was detected, which was found in seven patients with cleft palate, five patients with unilateral cleft lip and two patients with bilateral cleft lip. The association of facial cleft tissue with the inflammation process has previously been described in other studies (Seidel et al., 2022). Although samples were selected that should not have a pronounced inflammatory infiltrate, minimal infiltrate of inflammatory cells was found in these samples. This suggests that inflammation is a significant and a relatively common morphopathogenetic factor in facial cleft tissue, which, when interacting with other factors, such as cleft candidate genes, can make it difficult to assess postnatal morphopathogenesis of facial clefts.

BARX1 gene protein was not found in the epithelium in any patient, either in the control group or in all three cleft tissue groups, but statistically significant differences were identified in the connective tissue. In unilateral cleft lip connective tissue, BARX1 was rarely detected in general, in bilateral cleft lip connective tissue – rarely to a few positive cells, and in cleft palate connective tissue – rarely or not at all. In connective tissue, statistically significant differences in BARX1 immunoreactivity were found between the control group and the unilateral cleft lip group, between the unilateral cleft lip group and the

bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group. The results of our study suggest that BARX1 in connective tissue could be directly involved in the morphopathogenesis of a unilateral cleft lip postnatally, since it was in this patient group that the relative number of BARX1 positive connective tissue cells determined differed statistically significantly compared to both the control group and the bilateral cleft lip and cleft palate groups. *BARX1* gene in animals regulates the condensation of mesenchymal cells during head tissue development (Paudel et al., 2022), so it is possible that in humans, changes in the operation of the *BARX1* gene's coded protein could cause disturbances in the growth and regeneration of connective tissue, which could be involved in the morphopathogenesis of non-syndromic unilateral cleft lip.

Statistically significant differences in the immunoreactivity of the DLX4 gene protein between all study groups were found in both the epithelium and the connective tissue. In healthy tissues, DLX4 was generally not detected in both epithelial and connective tissue. In unilateral cleft lip tissue, DLX4 was rarely detected in both epithelial and connective tissue, in the epithelium of bilateral cleft lip tissue there were a few DLX4 positive cells, while in connective tissue – a rare occurrence, while in cleft palate tissue there was a rare occurrence of DLX4 positive epithelial cells and a rare occurrence of DLX4 positive cells in connective tissue. Statistically significant differences in the immunoreactivity were found in the epithelium between the unilateral cleft lip group and the cleft palate group and between the bilateral cleft lip group and the cleft palate group. However, no statistically significant differences in the epithelium were found between the control group and any of the cleft patient groups. Differences in DLX4 immunoreactivity in the epithelium between cleft patient groups suggest that *DLX4* gene protein could potentially be directly involved in the morphopathogenesis of cleft palate. However, there are conflicting data in the literature. A study in mice that had a loss of *DLX4* gene function concluded that

the *DLX4* gene was not necessary for epithelia growth and function, such as the epidermis, and the absence of the *DLX4* gene did not cause other significant pathologies in mice, which could be explained by the ability of other DLX genes to cover the functional role of the *DLX4* gene (Bhattacharya et al., 2018). Meanwhile, too much DLX4 activity is associated with accelerated proliferation of epithelial cells, impaired cell cycle function, and invasion of tumour cells, such as in nasopharyngeal carcinoma (Ling et al., 2020). In our study, the absence of a statistically significant difference compared to the epithelium of the control group could mean that the DLX4 protein in the epithelium is not, however, the most significant factor in the morphopathogenesis of facial clefts, although it cannot be excluded that there are slight differences in the immunoreactivity of DLX4 in the oral epithelium depending on the location of the acquired tissue (lip tissue or soft palate tissue).

In connective tissue, statistically significant differences in DLX4 protein immunoreactivity were found between the control group and the bilateral cleft lip group and between the control group and the cleft palate group. The study results suggest that the DLX4 protein in connective tissue could potentially be selectively involved in the morphopathogenesis of cleft palate and bilateral cleft lip, but not in the case of unilateral cleft lip. The effects of the DLX4 protein on connective tissue have not been extensively studied postnatally, but DLX4 in tumour tissue has previously been found to contribute to nitric oxide formation, which promotes endothelial cell growth and angiogenesis in tumour tissue (Trinh et al., 2015). DLX4 in bilateral cleft lip and cleft palate tissue could potentially regulate tissue vascularization, indirectly affecting facial tissue growth and the formation of orofacial cleft tissue.

The median number of cells containing FOXE1 in healthy tissues ranged from a few to a moderate number in the epithelium and a few in connective tissue. In the epithelium and connective tissue of the unilateral cleft lip patient

group there were a few FOXE1 containing cells. In bilateral cleft lip tissue, there were a few to moderate number of FOXE1 positive epithelial cells and a few FOXE1 positive connective tissue cells. In cleft palate tissue, there were a few FOXE1 containing cells in both the epithelium and connective tissue. Statistically significant differences in the immunoreactivity of the *FOXE1* gene protein between the control and patient groups were not found in the epithelium, but were detected in connective tissue. In connective tissue, statistically significant differences in FOXE1 immunoreactivity were determined between the control group and the unilateral cleft lip patient group, between the unilateral cleft lip group and the bilateral cleft lip group, and between the unilateral cleft lip group and the cleft palate group. The results of our study suggest that FOXE1 in connective tissue could potentially be associated with unilateral cleft lip morphopathogenesis due to significant differences compared to the control group and other groups of cleft tissue. In animals, the *FOXE1* gene regulates the development of the head and neck region by interacting with the SHH signalling pathway (Ratermans et al., 2023), and it is highly likely that the malfunction of FOXE1 in humans can contribute to the morphopathogenesis of facial clefts by interacting with multiple signalling pathways.

The median number of cells containing the HOXB3 gene protein in healthy tissue was a few in the epithelium and moderate in the connective tissue. In unilateral cleft lip tissue, HOXB3 was found in the epithelium to be moderate to numerous and in connective tissue moderate. In bilateral cleft lip tissue, HOXB3 was measured in moderate number in the epithelium and in a few to moderate number in connective tissue. In contrast, in cleft palate tissue, HOXB3 was generally found to be low to moderate in the epithelium and in moderate number in connective tissue. Statistically significant differences in the immunoreactivity of the HOXB3 gene protein were observed between the control and patient groups in both epithelial and connective tissue. In the

epithelium, statistically significant differences in the immunoreactivity of HOXB3 protein immunoreactivity were found between the control group and the unilateral cleft lip group, between the unilateral cleft lip group and the bilateral cleft lip group, and between the unilateral cleft lip group and the cleft palate group. In connective tissue, statistically significant differences in the immunoreactivity of HOXB3 protein immunoreactivity were found between the unilateral cleft lip group and the bilateral cleft lip group and between the unilateral cleft lip group and the cleft palate group. The results of the study suggest that postnatally HOXB3 in the epithelium and connective tissue could be a significant factor of unilateral cleft lip morphopathogenesis. The functional role of HOXB3 has been studied more in the case of malignant neoplasms (Zhu et al., 2023; Xue et al., 2018) since HOXB3 regulates cell proliferation. HOXB3 has been found to be capable of interacting with the WNT signalling pathway (Zhu et al., 2023; Yu et al., 2022), which is also significant for the development of the craniofacial region, which could be one of the possible explanations for why HOXB3 could be involved in the morphopathogenesis of unilateral cleft lip. The effect of HOXB3 on tissue growth has been more studied in tumours. The *HOXB3* gene can affect the cell cycle and can contribute to uncontrolled cell division, which has been seen in prostate cancer (Chen et al., 2013). There are also studies that conclude the exact opposite that the *HOXB3* gene has tumour suppressor gene activity in pancreatic cancer tissue, hindering tumour cell division and migration (Yang et al., 2016). Such conflicting information in the literature data, along with the findings of our study, could suggest that the effects of HOXB3 gene protein on tissue growth and formation could depend on the specific type of tissue, tissue location, and other factors such as the functioning of certain signalling pathways, which could be different in different types of cleft tissue.

MSX2 gene protein was not found in healthy oral cavity tissue, but in unilateral cleft lip tissue, MSX2 was measured in a moderate number in epithelial tissue and a few MSX2 positive cells were seen in connective tissue, in bilateral cleft lip tissue, MSX2 was detected in a few epithelial cells and a rare occurrence was observed in connective tissue, while in cleft palate tissue MSX2 was measured in moderate number in the epithelium and a rare occurrence was detected in connective tissue. Statistically significant differences in the immunoreactivity of the *MSX2* gene protein between control and patient groups were found both in the epithelial and connective tissue. In the epithelium, statistically significant differences in MSX2 protein immunoreactivity were found between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, between the control group and the cleft palate group, between the unilateral cleft lip group and the bilateral cleft lip group, and between the unilateral cleft lip group and the cleft palate group. Statistically significant differences in the immunoreactivity of the MSX2 protein in the connective tissue between the unilateral cleft lip group and the bilateral cleft lip group and between the unilateral cleft lip group and the cleft palate group. The results of our study suggest that MSX2 in the epithelium is postnatally involved in the morphopathogenesis of unilateral and bilateral cleft lip and cleft palate, but the most pronounced effect of MSX2 could be directly found in the tissue of unilateral cleft lip, as the relative number of MSX2 immunoreactive cells in the epithelium and connective tissue also differed statistically significantly from the other cleft tissue groups. In mice, the *MSX2* gene regulates the formation of the skull base (Nie, 2006). The absence or malfunction of the *MSX2* gene in mice causes ossification disorders and abnormalities in the development of the head region, including facial clefts (Satokata et al., 2000). In humans, MSX2 could potentially be involved in the morphopathogenesis of different facial clefts as a significant regulator of

craniofacial region growth and formation. The *MSX2* gene affects the formation and differentiation of the oral epithelium. The *MSX2* gene affects the development of the dental enamel organ in mice, preventing the external enamel epithelium from transforming into the stratified squamous oral cavity epithelium (Nakatomi et al., 2018). It is possible that changes in *MSX2* gene activity could be specific for humans in the epithelium affected by facial clefts, as demonstrated by the results of our study. A more pronounced finding of MSX2 was seen directly in the cleft-affected epithelium in all three cleft tissue groups and less pronounced in the connective tissue. The *MSX2* gene also affects tissue growth and cell division. The effect of MSX2 on tissue growth has been studied in colorectal cancer tissue (Liu et al., 2017), in which increased activity of the *MSX2* gene promotes growth and invasion of tumour cells, while loss of *MSX2* gene activity has the opposite effect, contributing to cell apoptosis and cell cycle arrest. The *MSX2* gene in mice regulates the differentiation of mesenchymal cells in certain areas of the head and neck that engage in oral connective tissue formation by interacting with the bone morphogenetic protein (BMP) signalling pathway (Sakagami et al., 2018). It is possible that changes in the relative number of MSX2 protein-containing cells detected in our study in cleft-affected connective tissue of the oral cavity compared to healthy tissue could also involve in cleft morphopathogenesis, affecting the formation and growth of cleft-affected connective tissue.

PAX7 gene protein in healthy oral cavity tissue was generally detected in a few to moderate number in both epithelial and connective tissue. In unilateral cleft lip tissue, PAX7 was found in moderate to numerous numbers in the epithelium and numerous in the connective tissue, in bilateral cleft lip tissue – moderate number in the epithelium and a few to moderate number in the connective tissue, while in cleft palate tissue – a few to moderate number in the epithelium and a moderate number in the connective tissue. Statistically

significant differences in the immunoreactivity of PAX7 gene protein between the control and patient groups were found in both epithelial and connective tissue. In the epithelium, statistically significant differences in the immunoreactivity of PAX7 protein were found between the control group and the unilateral cleft lip group, between the unilateral cleft lip group and the bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group. In connective tissue, statistically significant differences in the immunoreactivity of PAX7 protein were found between the control group and the unilateral cleft lip group, between the unilateral cleft lip group and the bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group. The results of the study suggest that PAX7 in both the epithelium and connective tissue may be postnatally involved primarily with the morphopathogenesis of the unilateral cleft lip. PAX7 is primarily described as a myogenesis regulator that interacts with the WNT signalling pathway during the craniofacial development process (Zhu et al., 2017; Nogueira et al., 2015). If PAX7 functions correctly in mice, PAX7 together with PAX3 can reduce the negative effects of teratogens and can prevent the development of facial abnormalities (Zalc et al., 2015). In the case of nonsyndromic unilateral cleft lip, PAX7 in humans could postnatally be one of the possible morphopathogenetic factors whose functional disorders could theoretically increase the effect of other factors, such as teratogens, on the formation of cleft lip tissue.

The median number of PAX9 gene protein in healthy oral cavity tissue was moderate in the epithelium, but the PAX9 gene protein was not found in healthy oral cavity connective tissue. In unilateral cleft lip tissue, PAX9 was generally found in a few to moderate number of epithelial cells and in moderate number in connective tissue. In bilateral cleft lip tissue, PAX9 was rarely detected in both epithelial and connective tissue, while in cleft palate tissue, PAX9 had a rare occurrence in the epithelium and had a few PAX9 positive cells

in the connective tissue. Statistically significant differences in the immunoreactivity of the *PAX9* gene protein were found between the control and patient groups in both epithelial and connective tissue. In the epithelium, statistically significant differences in the immunoreactivity of PAX9 protein were found between the control group and the bilateral cleft lip group, between the control group and the cleft palate group, between the unilateral cleft lip group and the bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group. In connective tissue, statistically significant differences in the immunoreactivity of PAX9 protein were found between the control group and the unilateral cleft lip group, between the control group and the cleft palate group, between the unilateral cleft lip group and the bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group, between the bilateral cleft lip group and the cleft palate group. The results of the study suggest that PAX9 in the epithelium could be more associated with the morphopathogenesis of bilateral cleft lip and cleft palate, while PAX9 in connective tissue could be more associated with unilateral cleft lip and cleft palate. PAX9 regulates epithelial differentiation in the oral cavity (Jonker et al., 2004) and regulates the morphogenesis of the secondary palate (Jia et al., 2020) by regulating the operation of the BMP and SHH signalling pathways (Zhou et al., 2013). The results of our study suggest that the different findings of the PAX9 gene protein in the cleft-affected epithelium and connective tissue of different clefts in humans could be associated with the formation of specific facial cleft phenotypes, such as the reduction of PAX9 in tissue could potentially contribute to the morphopathogenesis of a clinically more severe bilateral cleft lip phenotype. PAX9 has also been found to be involved in the regulation of tissue growth as it is able to limit cell division within tumours and promotes apoptosis (Liu et al., 2022). It could also suggest that changes in PAX9 activity can also potentially hinder the growth and formation of oral tissue in facial clefts.

RYK gene protein was rarely detected in healthy oral cavity tissue in both epithelial and connective tissue. In all cleft tissue groups (unilateral and bilateral cleft lip and cleft palate group) RYK was generally found to be in a moderate number both in the epithelium and connective tissue. Statistically significant differences in the immunoreactivity of the RYK gene protein between the control and patient groups were found in both epithelial and connective tissue. In the epithelium, statistically significant differences in immunoreactivity of the RYK protein were found between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, between the control group and the cleft palate group, between the unilateral cleft lip group and the cleft palate group. Statistically significant differences in RYK protein immunoreactivity were observed between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, and between the control group and the cleft palate group. The results of our study suggest that RYK in both epithelial and connective tissue may be involved in cleft morphopathogenesis in all three cleft patient groups. It is well known that RYK interacts with the WNT signalling pathway and that it regulates the polarity of epithelial cells (Macheda et al., 2012). RYK, along with the WNT signalling pathway, can also regulate other morphopathogenic processes such as inflammation, which has been described during the pulmonary development process in mice (Kim et al., 2022). Perhaps RYK's role in the morphopathogenesis of nonsyndromic orofacial clefts could be associated with the interference with the WNT signalling pathway, with other potential morphopathogenetic processes postnatally, such as inflammation, also being affected in parallel.

SHH gene protein in healthy oral cavity tissue was found in moderate to large numbers in the epithelium and a few to moderate number in connective tissue. In unilateral cleft lip tissue, SHH was generally found to be in moderate

number in both epithelial and connective tissue. In bilateral cleft lip tissue, SHH was detected in a few to moderate number in the epithelium and connective tissue, while cleft palate tissue contained a few SHH positive epithelial cells and low to moderate number of SHH positive connective tissue cells. Statistically significant differences in SHH gene protein immunoreactivity were found between the control and patient groups in both epithelial and connective tissue. In the epithelium, statistically significant differences in SHH protein immunoreactivity were found between the control group and the bilateral cleft lip group, between the control group and the cleft palate group, between the unilateral cleft lip group and the bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group, between the bilateral cleft lip group and the cleft palate group. The results of our study suggest that SHH in the epithelium could be more associated with bilateral cleft lip and cleft palate formation. The association of SHH with a bilateral cleft lip could signal the involvement of this protein in the development of a clinically more severe phenotype in humans. SHH has previously been described as the regulatory factor for upper lip morphogenesis (Everson et al., 2017). The involvement of SHH in the morphopathogenesis of the cleft palate in animals has already been previously described (Shine et al., 2019).

In connective tissue, statistically significant differences in the immunoreactivity of the SHH protein were found between the control group and the cleft palate group, between the unilateral cleft lip group and the bilateral cleft lip group, and between the unilateral cleft lip group and the cleft palate group. The results of the study suggest that SHH in connective tissue may be more associated with cleft palate, but there are also statistically significant differences between unilateral and bilateral cleft lip in the immunoreactivity of SHH within connective tissue. The role of SHH in the morphopathogenesis of the cleft palate has previously been described in mice, where increased activity of the SHH

signalling pathway is associated with the formation of the cleft palate (Hammond et al., 2018), but the results of our study suggest that the relative number of SHH positive cells has been reduced the tissue of the in cleft palate. Reduced SHH signalling pathway activity in mice in the upper jaw mesenchyme usually increases the function of the BMP signalling pathway, which in turn increases connective tissue formation in the developing oral cavity, especially in the maxillary region and the palate (Xue et al., 2019). Because our study analysed tissue affected by different orofacial clefts already after the completion of the mesenchymal differentiation and the cleft tissue formation process, it is possible that changes in the relative amount of SHH in cleft tissue could be postnatally influenced by other factors, such as changes in the activity of other signalling pathways in the connective tissue of the oral cavity. In mice, SHH signalling has been determined to interact with the TGFβ signalling pathway in later stages of secondary palate development, when mesenchymocytes have already differentiated into fibroblast-like cells, by using paracrine signalling to affect connective tissue growth in the palate (Ohki et al., 2020). Similar interactions, which would depend on tissue differentiation and degree of maturity, could also exist in tissues affected by human orofacial clefts, but more studies would be needed to assess the exact changes in how SHH and other signalling pathways function in oral mucosa tissue depending on the age of individuals, which could be problematic for ethical reasons.

SOX3 gene protein was detected in healthy oral mucosal tissue in numerous to abundant numbers in the epithelium and in numerous numbers in the connective tissue. In unilateral cleft lip tissue, SOX3 was generally found in moderate to numerous numbers in the epithelium and in moderate numbers in the connective tissue, in bilateral cleft lip tissue – in a moderate number in both the epithelial and connective tissue, while in cleft palate tissue – in few to moderate numbers in the epithelium and in moderate numbers in the connective

tissue. Statistically significant differences were found in the immunoreactivity of the *SOX3* gene protein between the control and patient groups in both epithelial and connective tissue. In the epithelium, statistically significant differences were found in the immunoreactivity of the SOX3 protein between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, between the control group and the cleft palate group, between the unilateral cleft lip group and the bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group. In connective tissue, statistically significant differences in SOX3 protein immunoreactivity were found between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, between the control group and the cleft palate group. The results of our study suggest that SOX3 postnatally in both epithelial and connective tissue could be involved in the morphopathogenesis of unilateral and bilateral cleft lip and cleft palate. Also notable is the statistically significant difference in SOX3 immunoreactivity in the epithelium of unilateral cleft lip when compared to the other orofacial cleft groups that could associate SOX3 in the epithelium more with the unilateral cleft lip. The role of the *SOX3* gene in cleft morphopathogenesis is relatively uncertain. The *SOX3* gene has been studied in tumours where increased expression of the *SOX3* gene contributes to epithelial-mesenchymal transition, cell migration, and tumour cell invasion (Qie et al., 2017). SOX3 functionality has been studied in animals and it is necessary for spermatogonia differentiation in mice prior to reaching sexual maturity, but not in adult mice (Laronda and Jameson, 2011). SOX3 could potentially be involved in cleft morphopathogenesis by affecting cleavage tissue formation. SOX3 functionality could also be age dependent in human facial cleft tissue, but it would be necessary to evaluate the immunoreactivity of SOX3 in human tissue from different age groups, which is problematic for ethical reasons.

The WNT3A gene protein in healthy oral cavity tissue was found in numerous to abundant number in the epithelium and in numerous numbers in the connective tissue, in unilateral cleft lip tissue – a few positive cells for WNT3A were found both in the epithelium and connective tissue, in bilateral cleft lip tissue – a rare occurrence in the epithelium and a few in the connective tissue, while in cleft palate tissue – a rare occurrence both in the epithelium and connective tissue. Statistically significant differences were found in the immunoreactivity of the WNT3A gene protein between the control and patient groups in both epithelial and connective tissue. In the epithelium, statistically significant differences in WNT3A protein immunoreactivity were found between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, between the control group and the cleft palate group, between the unilateral cleft lip group and the bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group. In connective tissue, statistically significant differences in WNT3A protein immunoreactivity were found between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, between the control group and the cleft palate group. The results of the study suggest that, as with SOX3, WNT3A in both connective tissue and epithelium could be postnatally associated with the morphopathogenesis of facial clefts in the three patient groups, but perhaps a more significant role could be played directly in the case of unilateral cleft lip. Increased WNT3A activity is associated with abnormalities that increase oral keratinocyte division activity, such as oral epithelial dysplasia or oral squamous cell carcinoma due to increased WNT signalling pathway activity (Reyes et al., 2019). The results of the study suggest that decreased WNT3A activity in facial cleft tissue could postnatally affect epitheliocyte growth and therefore also cleft morphopathogenesis.

The WNT9B gene protein in healthy oral mucosa tissue was found in numerous to abundant number in both the epithelium and connective tissue, in unilateral cleft lip tissue – in numerous to abundant number in the epithelium and in numerous number in the connective tissue, in bilateral cleft lip tissue – in moderate number in the epithelium and in moderate to numerous number in the connective tissue, while in cleft palate tissue – in moderate number in the epithelium and in moderate to numerous number in the connective tissue. Statistically significant differences were found in the immunoreactivity of the WNT9B gene protein between the control and patient groups in both epithelial and connective tissue. In the epithelium, statistically significant differences in WNT9B protein immunoreactivity were found between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, between the control group and the cleft palate group, between the unilateral cleft lip group and the bilateral cleft lip group, between the unilateral cleft lip group and the cleft palate group. In connective tissue, statistically significant differences in WNT9B protein immunoreactivity were found between the control group and the unilateral cleft lip group, between the control group and the bilateral cleft lip group, between the control group and the cleft palate group, and between the unilateral cleft lip group and the cleft palate group. The results of our study suggest that WNT9B in both epithelial and connective tissue could be a significant morphopathogenetic factor in unilateral cleft lip, bilateral cleft lip, and cleft palate tissue. The immunoreactivity of WNT9B immunoreactivity in the epithelium and connective tissue in unilateral cleft lip tissue differed statistically significantly from other facial cleft groups. In mice, the *WNT9B* gene participates in the classic WNT signalling pathway and regulates the upper lip fusion process (Lan et al., 2006). Similar WNT9B interactions could also be common in humans, which could be a relatively significant morphopathogenetic mechanism in craniofacial clefts.

A correlation analysis between cleft candidate gene proteins in control and cleft patient groups showed several statistically significant correlations. Several statistically significant correlations between the evaluated cleft candidate genes were identified in both control groups, but these should be carefully evaluated due to the low number of patients in each control group, which may increase the probability of statistical randomness.

A statistically significant strong positive correlation was observed between the age of the patients and the relative number of RYK positive cells in the connective tissue in the first control group (patients who underwent plastic surgery of the upper lip frenulum plastic surgery). This correlation may be accidental due to the relatively small number of control patients, but there could probably be a relationship between the relative number of RYK positive cells and the age of the patients in relatively healthy oral tissue. Currently, there are no studies on the changes in the relative amount of RYK protein in oral mucosa tissue and the relation to the age of the individuals. The functionality of RYK protein has been studied in oncology because the increase in RYK protein is associated with increased WNT signalling activity, which can contribute to more active cell growth and invasion in tumours (Kim et al., 2015; Habu et al., 2014). RYK could potentially be involved in normal oral mucosal connective tissue growth.

In the first control group, several statistically significant very strong correlations between cleft candidate gene proteins were calculated. Four positive correlations primarily involved DLX4 in epithelium and connective tissue. DLX4 was also found in two statistically significant negative correlations. In statistically significant positive correlations, PAX7 involvement was observed in three cases. The results of our study suggest that DLX4 and PAX7 could be active factors in relatively healthy oral tissue, which participates in the maintenance and shaping of oral mucosal tissue. DLX4 is involved in the

regulation of epithelial tissue growth in the head region and has been studied in association with oncological diseases (Ling et al., 2020), while PAX7 has been more studied in relation to its functional role in myogenesis (Collins et al., 2009). Naturally, the tissue in the first control group is not entirely healthy, as changes that could be caused by upper lip frenulum hypertrophy, which was the necessary indication to obtain relatively healthy oral mucosal tissue, cannot be ruled out.

A statistically significant strong correlation was observed between SHH in the epithelium and SHH in the connective tissue in the second control group (patients whose oral tissue was obtained from the historical collection of RSU AAI historical collection). This suggests that SHH is a relatively active factor in relatively healthy oral mucosal tissue of newborns. The SHH signalling pathway is necessary for the oral mucosa and the dental development process by regulating the interaction between the oral epithelium and connective tissue (Sagai et al., 2017).

Many statistically significant correlations were found in all three cleft patient groups. The large number of correlations suggests a complicated interaction between the evaluated cleft candidate genes in the morphopathogenesis of orofacial clefts postnatally.

In unilateral cleft lip group, a hundred and four statistically significant positive correlations were determined between almost all of evaluated cleft candidate genes in both epithelial and connective tissue, excluding BARX1. In unilateral cleft lip tissue group the protein most involved in statistically significant strong positive correlations (r_s = 0.6–0.8) was HOXB3 in epithelium (three times) and WNT9B in epithelium (two times). This suggests that HOXB3 and WNT9B could be more significant factors in the morphopathogenesis of unilateral cleft lip in the epithelium than other cleft candidate genes of the ones studied. HOXB3 has been studied as a factor that affects the growth of epithelial and connective tissues in the cranial region (Zhang et al., 2021), while WNT9B

in mice is directly involved in the formation of the upper lip (Lan et al., 2006). In unilateral cleft lip group, the proteins most involved within statistically significant moderate positive correlations (r_s = 0.4–0.6) were HOXB3 in the epithelium (ten times), PAX9 in the epithelium (nine times), PAX7 in connective tissue, PAX9 in connective tissue, and SOX3 in epithelium (eight times each). This further demonstrates that HOXB3 could be a significant postnatal morphopathogenetic factor for unilateral cleft lip formation. PAX9 has been studied as a factor that regulates the differentiation of the oral epithelium and mucosal tissue (Jonker et al., 2004), so it could also participate in the morphopathogenesis of unilateral cleft lip. SOX3 has not been extensively studied in the oral epithelium, but in mice it regulates the development of brain and cranial structures (Adikusuma et al., 2017). SOX3 possibly could be involved in the morphopathogenesis of unilateral cleft lip postnatally. In unilateral cleft lip group, the proteins most involved in statistically significant weak positive correlations (r_s = 0.2–0.4) were HOXB3 in the epithelium, MSX2 in connective tissue and SHH in connective tissue (six times each). MSX2 is involved in the regulation of oral mucosal tissue growth, such as suppressing tumour stem cell formation in squamous cell carcinoma (Keyimu et al., 2021). MSX2 could also be involved in the morphopathogenesis of unilateral cleft lip, potentially inhibiting mucosal tissue growth and contributing to the formation of cleft phenotypes. The malfunction of SHH, meanwhile, has previously been linked to the development of a cleft lip (Everson et al., 2017), which also matches the results of our study.

Seventy-two statistically significant positive correlations were calculated between the evaluated cleft candidate genes in the bilateral cleft lip group. In bilateral cleft lip group, the proteins most involved in the statistically significant very strong positive correlations (r_s = 0.8–1.0) group were HOXB3 in the epithelium, PAX9 in the epithelium, SHH in the epithelium and WNT9B in

epithelium (twice each) similarly to the unilateral cleft lip group. This suggests that the morphopathogenetic mechanisms in both unilateral and bilateral cleft lip tissue could be similar, which could be attributed to both cleavages affecting the upper lip tissue. In bilateral cleft lip group, the proteins involved in statistically significant strong positive correlations (r_s = 0.8–1.0) were FOXE1 in the epithelium, FOXE1 in connective tissue, SHH in connective tissue, and WNT3A in epithelium (each once). FOXE1 in epithelium was strongly correlated with FOXE1 in connective tissue, suggesting that this protein is actively involved in the formation of the oral epithelium and connective tissue in bilateral cleft lip, a clinically more severe form of cleft lip than unilateral cleft lip. FOXE1 is considered a significant cleft candidate gene in the morphopathogenesis of both cleft lip and palate (Moreno et al., 2009). WNT3A is more likely to participate in the WNT signalling pathway to regulate mucosal tissue homeostasis in tissue affected by clefts. Studies have shown that oral epithelial cells respond to the WNT signalling pathway after being damaged, which promotes basal cell proliferation and epithelial tissue recovery (Yuan et al., 2019). In the bilateral cleft lip tissue group, the most commonly involved proteins in statistically significant strong positive correlations (r_s = 0.6–0.8) were FOXE1 in connective tissue (eleven times) as well as FOXE1 in epithelium, MSX2 in epithelium, MSX2 in connective tissue, SOX3 in epithelium (nine times each). This suggests that FOXE1 both in epithelial tissue and in connective tissue could be an active morphopathogenetic factor of bilateral cleft lip tissue postnatally. FOXE1 regulates the correct growth of epithelial tissue, which has been described in studies evaluating the effects of FOXE1 on hair follicle growth (Brancaccio et al., 2004), suggesting that FOXE1 could also interfere with epithelial growth in bilateral cleft lip tissue. The involvement of MSX2 and SOX3 in the correlations within the bilateral cleft lip group is similar to the unilateral cleft lip group, while MSX2 and SOX3 are involved in stronger correlations than in the

unilateral cleft lip group, suggesting morphopathogenetic differences in tissue between the two types of cleft lip. In bilateral cleft lip group, the proteins most commonly involved in statistically significant moderate positive correlations (r_s = 0.6–0.8) were SHH in the epithelium (three times), DLX4 in the epithelium, FOXE1 in the epithelium, SOX3 in connective tissue, WNT3A in the epithelium (two times each), which is similar to the unilateral cleft lip group, further demonstrating morphopathogenic similarities between the two cleft lip phenotypes.

Seventy-one statistically significant positive correlation between the evaluated candidate cleavage genes was calculated in the cleft palate group. A statistically significant very close positive correlation (r_s = 0.8–1.0) between SOX3 in the epithelium and WNT9B in the epithelium (r_s = 0.862, p = < 0.001) was determined in the cleft palate group. The interaction between SOX3 and the WNT signal pathway has previously been described in animal studies (Zhang et al., 2003; Zorn et al., 1999), which found that SOX3 is capable of binding to β-catenin, which in turn regulates the activity of the WNT signalling pathway. In cleft palate tissue, SOX3 and WNT9B interactions could be one of the morphopathogenetic mechanisms specific to this type of cleft. In the cleft palate group, the main proteins involved in statistically significant strong positive correlations (r_s = 0.6–0.8) were PAX9 in connective tissue (four times), HOXB3 in the epithelium, PAX7 in the epithelium, PAX7 in connective tissue (three correlations each), while in statistically significant moderate positive correlations (r_s = 0.4–0.6) the most commonly involved proteins were SHH in connective tissue (twelve times), PAX9 in the epithelium (nine times), PAX7 in connective tissue (eight times) and WNT3A in connective tissue (eight times). This is similar to the unilateral and bilateral cleft lip group. The involvement of cleft candidate genes of similar correlations in all three cleft groups suggests that there are relatively similar morphopathogenetic processes in tissues of both the

cleft lip and the cleft palate. In the group of cleft palate tissue, the main protein involved in statistically significant weak positive correlations (r_s = 0.2–0.4) was RYK (twice in connective tissue and once in epithelium). Mutations in the *RYK* gene have previously been associated with the cleft lip and cleft palate (Watanabe et al., 2006), which confirms that the malfunction of the RYK protein could be part of the morphopathogenetic mechanisms of the cleft palate, most likely interacting with the WNT signalling pathway (Macheda et al., 2012).

Although all cleft candidate genes showed statistically significant differences in immunoreactivity between the control group and cleft patient groups, individual cleft candidate genes were more associated with certain cleft phenotypes. Unilateral cleft lip tissue has a more pronounced involvement of HOXB3, FOXE1, BARX1 and PAX7, bilateral cleft lip and cleft palate tissue – DLX4, PAX9, SHH, but all three cleft phenotypes – MSX2, RYK, SOX3, WNT3A and WNT9B.

Conclusions

1. Vacuolization of the oral epithelium and proliferation of basal cells are considered nonspecific compensatory tissue changes in the case of facial clefts. Relatively common inflammation is an accompanying factor in the morphopathogenesis of clefts.
2. Among the cleft candidate genes evaluated, changes in the relative number of cells containing gene proteins selectively characterize either healthy or cleft-affected oral mucosa tissue. DLX4, FOXE1, HOXB3, PAX7, RYK, SHH, SOX3, WNT3A and WNT9B gene proteins are found in healthy oral mucosa tissue: in relatively greater amounts WNT9B, WNT3A, SOX3 gene proteins can be found, in moderate number – SHH, PAX7, HOXB3, FOXE1, less – PAX9, DLX4, RYK, while virtually nothing can be found in the case of BARX1 and MSX2 gene proteins. The absence of BARX1 and MSX2 in healthy but not cleft-affected tissue suggests an increase in these gene proteins only in cleft morphopathogenesis. The statistically lower expression of SOX3, WNT3A, and WNT9B only in cleft tissue indicates a decrease in the expression of these genes as also characteristic of the overall cleft morphopathogenesis process. Finally, PAX9 and RYK are also reported without statistically significant differences but with a tendency to decrease in cleft-affected tissue.
3. Some gene proteins lack selective increase in the case of certain types of clefts. Thus, it is the intense expression of BARX1, FOXE1, HOXB3 and PAX7 that characterises a unilateral cleft, pointing to tissue, especially connective tissue, cell regeneration and intensification of proliferation potential supported by a series of statistically significant differences between these factors.

4. The phenotype of a bilateral cleft is characterised by changes in the expression of DLX4, PAX9, and SHH, with the first two showing increased expression and SHH showing reduced expression. The selective release of these factors not only justifies their involvement in the morphopathogenesis of a bilateral cleft, but also highlights the main compensatory stimulated functions in tissue – the vascularization of connective tissue in case of DLX4, growth regulation by PAX9, and the formation of the most severe cleft type in case of SHH.
5. All cleft phenotypes are characterised by increases in MSX2 and RYK, while reductions are observed for SOX3, WNT3A and WNT9B in tissue. Without the combined universal effect, the changes of MSX2 could selectively disrupt connective tissue growth and differentiation, the RYK changes – the operation of the WNT signalling pathway, SOX3, WNT3A, and WNT9B – the growth and differentiation of all evaluated types of cleft tissue.
6. Similar correlations between the expression of selected gene proteins in all cleft phenotypes suggest similar mechanisms of morphopathogenesis by determining only the phenotype of gene proteins specific to clefts. The correlations between HOXB3 and WNT9B in the unilateral cleft lip group, between HOXB3, PAX9, SHH and WNT9B in the epithelium of the bilateral cleft lip group, and between SOX3 and WNT9B in the epithelium of the cleft palate group are particularly important.

Proposals for research in the future

Cleft candidate genes are a very broad group of cleft morphopathogenetic factors that are involved in the regulation of tissue development and maturation in the facial region. This study looks at **only a fraction of known cleft candidate genes and their encoded proteins**, so further research on other similar genes and their proteins could be a significant research direction in cleavage morphopathogenesis.

Cleft morphopathogenesis is a complex process that involves not only cleft candidate genes but also inflammation, growth, and tissue remodelling factors. Interactions between these factors make it difficult to find common mechanisms of cleft morphopathogenesis. **Comparisons between groups of factors, such as cleft candidate genes and inflammatory markers in tissue affected by clefts**, could enhance our understanding of the growth and formation of facial cleft tissue in humans. This could provide an opportunity to develop less invasive treatment methods or even cleft prevention techniques, tailored to the knowledge of facial cleft morphopathogenesis.

Given the interaction of complex factors in tissues affected by facial clefts, future morphological studies in the assessment of cleft morphopathogenesis could include not only immunohistochemistry, but also other complementary research methods such as *in situ* hybridisation. This could improve existing knowledge not only on the expression of cleft candidate gene proteins in tissue, but **also on the transcriptome, or on genome peculiarities in nonsyndromic orofacial clefts**.

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

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

1. **Vaivads, M.**, Akota, I., Pilmane, M. 2023. Characterization of SHH, SOX3, WNT3A and WNT9B Proteins in Human Non-syndromic Cleft Lip and Palate Tissue. Dentistry Journal, 11(6), 151. https://doi.org/10.3390/dj11060151

2. **Vaivads, M.**, Akota, I., Pilmane, M. 2022. Immunohistochemical Evaluation of BARX1, DLX4, FOXE1, HOXB3, and MSX2 in Nonsyndromic Cleft Affected Tissue. Acta medica Lituanica, 29(2), 216–239. https://doi.org/10.15388/Amed.2022.29.2.13

3. **Vaivads, M.**, Akota, I., Pilmane, M. 2021. PAX7, PAX9 and RYK expression in cleft affected tissue. Medicina (Lithuania), 57(10), 1075. https://doi.org/10.3390/medicina57101075

4. **Vaivads, M.**, Akota, I., Pilmane, M. 2021. Cleft Candidate Genes and Their Products in Human Unilateral Cleft Lip Tissue. Diseases, 9(2), 26. https://doi.org/10.3390/diseases9020026

Publications included in international scientific journals, including university article collections

1. **Vaivads, M.**, Balode, E., Pilmane, M. 2020. Factors affecting facial development and formation of cleft lip and palate: a literature review. Papers on Anthropology, 29(2), 22–35. https://doi.org/10.12697/poa.2020.29.2.02

Reports and theses at international congresses and conferences

1. **Vaivads, M.**, Akota, I., Pilmane, M. 2023. Cleft Candidate Gene Immunoreactivity in Human Non_Syndromic Cleft Affected Tissue. Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice" 29.–31.03.2023. Oral presentation.

2. **Vaivads, M.**, Akota, I., Pilmane, M. 2023. Evaluation of sonic hedgehog, sex-determining region Y-box transcription factor 3 and wingless-type family member 3A and 9B in different cleft tissue. Practical Ophthalmology. Medical and Environmental Problems of our Days: Collection of Works International Scientific and Practical Interdisciplinary Conference. State Institution "National Research Center for Radiation medicine of the National Academy of Medical Sciences of Ukraine", 12–13. Oral presentation.

3. **Vaivads, M.**, Akota, I., & Pilmane, M. 2022. PAX7, PAX9 and RYK expression in cleft affected tissue. 10th Congress of Baltic Association for Maxillofacial and Plastic Surgery and 16th Joint Symposium Riga–Rostock, Rīga, Latvia, 30. Poster presentation.

4. **Vaivads, M.**, Pilmane, M., Akota, I. 2021. Cleft candidate genes and their products in human unilateral cleft lip tissue. Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts, 24.–26.03.2021, 405. Poster presentation.

5. Goida, J., **Vaivads, M.**, Pilmane, M. 2021. Tissue indicators of inflammation in the cleft affected tissue. Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts, |24.–26.03.2021, 429. Poster presentation.

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