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Detecting Early Signs of Malignancy of Oral Leukoplakia in its Tissues and Saliva

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

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Sub-Sector – Dentistry

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

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Secretary of the Promotion Council:

Dr. med., Assistant Professor **Ilze Šalma**

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Abbreviations used in the Thesis

CDKN1B	Cyclin-dependent kinase inhibitor 1 B
HPV	Human papillomavirus
DNA	Deoxyribonucleic acid
DOI	Depth of invasion
EM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EUCAN	European Union and Canada
HPVOED	HPV-associated oral epithelial dysplasia
G_1	Phase of the cell cycle
G1	Well differentiated carcinoma
G2	Moderately differentiated carcinoma
G3	Low differentiated carcinoma
MT	Malignant transformation
NOS	Not otherwise specified
OSCC	Oral squamous cell carcinoma
OL	Oral leukoplakia
OPMD	Oral potentially malignant disease
RNA	Ribonucleic acid
SolCD44	Soluble CD44
TP	Total protein
WHO	World Health Organisation

Introduction

Oral squamous cell carcinoma (OSCC) tends to develop against a background of oral potentially malignant diseases (OPMD), which include leukoplakia, erythroplakia, submucous fibrosis and erosive and atrophic forms of oral *lichen ruber planus* (Renaud-Vilmer et al., 2017; Sundberg et al., 2019). In the general population, oral leukoplakia occurs in approximately 3.41 % to 11.74 % of people (Zhang et al., 2023). In Northern European countries, as well as in Latvia (Miluna et al., 2022; Warnakulasuriya et al., 2021), in recent years, young people are increasingly using smokeless tobacco, which, along with alcohol use, smoking and chronic inflammatory processes of the oral mucosa, are potential causes of precancerous lesions and oral malignancies (Leon et al., 2016). The role of oral leukoplakia (OL) in the development of squamous cell carcinoma has been discussed in the literature for many decades (Garcia et al., 2019; Roye et al., 1996; Wang et al., 2009). There have been attempts to demonstrate other risk factors for malignant transformation of oral leukoplakia to carcinoma: for example, leukoplakia of the tongue or the base of the mouth that are larger than 2 cm in size and represent a non-homogeneous type of OL (Assimakopoulos et al., 2002; Speight et al., 2018).

Oral leukoplakia is described as the most common oral potentially malignant disease (Iocca et al., 2020), transforming into oral squamous cell carcinoma between 1 and 12 % of cases in different countries worldwide (Narayan et al., 2016; Warnakulasuriya, 2018), although there are studies in the Eastern countries reporting higher transformation rates of up to 34 % (Ganesh et al., 2018; Saldivia-Siracusa et al., 2021). However, it should be stressed that not all leukoplakia transform into malignancies (Iocca et al., 2020; Kuribayashi et al., 2015). The 'gold standard' for the risk of malignant transformation includes the assessment of epithelial dysplasia (van der Waal, 2009).

The histopathological classification of oral epithelial dysplasia was revised several times between 1978 and 2017, and in 2017 the World Health Organization (WHO) approved three grades of dysplasia: mild, moderate and severe, which were combined with cancer *in situ* (El-Naggar et al., 2017). Despite the fact that more severe degrees of dysplasia are associated with a higher risk of transformation into carcinoma, mild dysplasias can also progress to cancer (Arnaoutakis et al., 2013), while some severe dysplasias do not progress to carcinoma regardless of environmental factors (Nankivell et al., 2013). However, the histopathological classification of oral epithelial dysplasia was revised again in 2023, with 2 grades, mild and severe (WHO, 2024).

Several studies have shown that oral leukoplakia transforms into squamous cell carcinoma in approximately 55–57 months (Bukovszky et al., 2023; Cerqueira et al., 2021), therefore their investigation is of great importance. It should be emphasised that 4 % of all oncological deaths are due to oral, pharyngeal and laryngeal carcinoma. It is the eighth most common cause of death from carcinoma worldwide. In the Baltic countries, the 5-year survival rate for oral squamous cell carcinoma ranges from 19.74 % in Estonia to 37.98 % in Latvia (Anonymous, 2020).

From the point of view of normal histology, it is important to emphasise that the migration rates of epithelial cells in the oral cavity vary: its stem cells reach the surface of the buccal mucosa in 25 days, in the gingiva in 50 days, and in the junction areas in a rapid 5–6 days (Nanci, 2017). Thus, the biopsy captures only a single moment in the complex process of mucosal regeneration, hyperplasia, dysplasia and malignisation.

Animal models are not always acceptable to understand this long and complex transformation from leukoplakia to invasive carcinoma (Herold-Mende et al., 1996; Wielenga et al., 1993). The process of malignant neoplasia formation undoubtedly has multiple subtle mechanisms at the level

of genes, molecules and even nanoparticles (Zlotogorski-Hurvitz et al., 2015). Combined immunohistochemical, electronmicroscopic, genetic and immunological studies are needed to understand the complex mechanisms of malignancy in the oral mucosa. It should be noted that intensive efforts are ongoing to identify certain prognostic biomarkers that could indicate early malignant transformation of oral leukoplakia. The detection of cytoplasmic and nuclear antigens in oral mucosal epithelial leukoplakia tissues provides insight into the molecular mechanisms of its possible malignant transformation. Important OL research applications include the combined analysis of the epithelial cycle regulator p27 and the transcriptional protein ThPOK, as well as tumour stem cell CD44 and exosomal CD9 antigens. The immunohistochemical characterisation of these proteins in the literature is scarce, fragmented and controversial.

Aim of the Thesis

To identify early signs of oral leukoplakia malignisation by assessing clinical, morphological and immunohistochemical changes in different types of leukoplakia in the oral cavity in conjunction with salivary test parameters.

Tasks of the Thesis

1 To compile epidemiological, clinical laboratory and morphological data on oral leukoplakia patients and to evaluate them;
2 To analyse the VELscope fluorescence spectroscopy data and their relevance for the delineation of pathologically altered oral tissues and biopsy site;
3 To determine and evaluate the expression data of soluble CD44 and total protein in saliva of persons with healthy mucosa, various forms of oral mucosal leukoplakia and squamous cell carcinoma;

4 To analyse the frequency, type and localisation of immunohistochemical expression of cell membrane CD44 and CD9 antigens in oral leukoplakia compared to healthy mucosal tissues and squamous cell carcinomas;

5 To determine and evaluate the frequency, pattern and localisation of expression of the cell nuclear immunohistochemical markers ThPOK and p27 in oral leukoplakia compared to healthy mucosa and squamous cell carcinomas;

6 To identify the most informative potential malignancy criteria for oral leukoplakia.

Hypotheses of the Thesis

1 SolCD44 expression in saliva shows an increased presence of CD44 protein in the saliva of non-homogeneous leukoplakia patients with potentially early signs of malignancy.

2 Changes in the intracellular localisation of oral mucosal epithelial membrane (CD44, CD9) and nuclear (p27, ThPOK) antigens indicate early malignant changes in leukoplakia tissue.

Novelty of the Thesis

1 In this study, for the first time in Latvia and the Baltics, the salivary soluble CD44 and the total protein expression in patients with oral leukoplakia were assessed and compared with CD44 expression in leukoplakia tissues. The development of early malignancy criteria for oral leukoplakia is of global scientific importance as the search for these signs to improve diagnosis and treatment, as well as to assess the prognosis of the disease, is ongoing. The salivary soluble CD44 test is a convenient and easy-to-use test for the determination of the risk of oral malignancy in dental practice.

2 For the first time, oral squamous epithelial cell membrane and nuclear biomarkers were analysed regionally in homogeneous and non-homogeneous leukoplakia, looking for analogous changes with oral squamous carcinomas, to assess their prognostic significance in carcinogenesis.

Personal contribution

The author of the Thesis carried out the planning of the research process, literature collection and analysis, processing of clinical data of patients.

The OncAlert saliva test and the oral mucosa of the patients were also evaluated. Photographs were taken with a Velscope device. The author of the Thesis selected immunohistochemical markers based on literature data on oral mucosal leukoplakia and squamous cell carcinomas and analysed the results of immunohistochemical reactions in histological samples. In cooperation with the Statistical Laboratory of RSU (Learning Laboratory of Statistics), the author performed statistical data processing.

Ethical aspects of the work

The study was conducted in accordance with the Declaration of Helsinki and the Permission No 3 / 18.08.2016 of the Ethics Committee of Rīga Stradiņš University. All patients were informed about the study and signed an informed consent form. Each phase of the study was conducted in accordance with ethical principles.

1 Materials and methods

Place of performance

1 Rīga Stradiņš University Institute of Stomatology, Centre of Oral Medicine;
2 Pauls Stradiņš Clinical University Hospital, Centre for Oral and Maxillofacial Surgery;
3 Pauls Stradiņš Clinical University Hospital, Institute of Pathology.

Material and technical resources

The study was carried out using the technical equipment of the RSU Institute of Dentistry, Centre of Oral Medicine, PSCUH Centre of Oral and Maxillofacial Surgery and the Institute of Pathology. The financial support was obtained within the framework of the Rīga Stradiņš University doctoral study grant and project No 8.2.2.0/I/004 “Support for Doctoral Students' Involvement in Scientific Research and Study”.

1.1 Characteristics of the study groups

1.1.1 Study group of patients with oral leukoplakia

We included data of 50 oral leukoplakia patients who were consulted and treated at the RSU Institute of Dentistry, Oral Medicine Centre and in patient unit, as well as operated at the Oral, Maxillofacial Surgery Centre of P. Stradiņš Clinical University Hospital from 2016 to 2022. First, the author of the Thesis clinically assessed the leukoplakia variant in each patient – homogeneous or non-homogeneous, then we determined the OL subtype – simplex, nodular, verrucous or erythroleukoplakia. We analysed the localisation and size of the oral leukoplakia and the age and sex of the patients.

1.1.2 Comparative group of oral squamous cell carcinomas

The comparative group included 20 patients with oral mucosal squamous cell carcinoma who were treated at the RSU Institute of Dentistry, Centre of Oral Medicine, between 2016 and 2022. Carcinomas were analysed according to the WHO classification, taking into account TMN values. The grades of differentiation of squamous cell carcinoma were Grade 1 (G1, well differentiated carcinoma), Grade 2 (G2, moderately differentiated carcinoma), Grade 3 (G3, low differentiated carcinoma). Clinical stages of squamous cell carcinoma were graded from I to IV-c.

The Thesis was developed before the publication of the new WHO book (Head and Neck Tumours, 5th edition, 2023), so we did not include data on HPV-positive and negative dysplasias and oral mucosa carcinomas, which were not determined in clinics, and we used the NOS (not otherwise specified) classification of squamous cell carcinomas without considering the DOI (depth of invasion below basement membrane) (WHO, 2024).

1.1.3 Comparative group of healthy oral mucosa

The second comparison group consisted of 20 patients in whom, after elliptical excision of *epulis fissuratum* and fibropapillomas, their distal and proximal mucosal regions were used as whole mucosa samples if they were visually intact. The presence of healthy mucosa was confirmed by microscopic evaluation. Healthy mucosa was considered as mucosa with histologically normal structure.

1.2 Study design

The study design is presented in Figure 1.1.

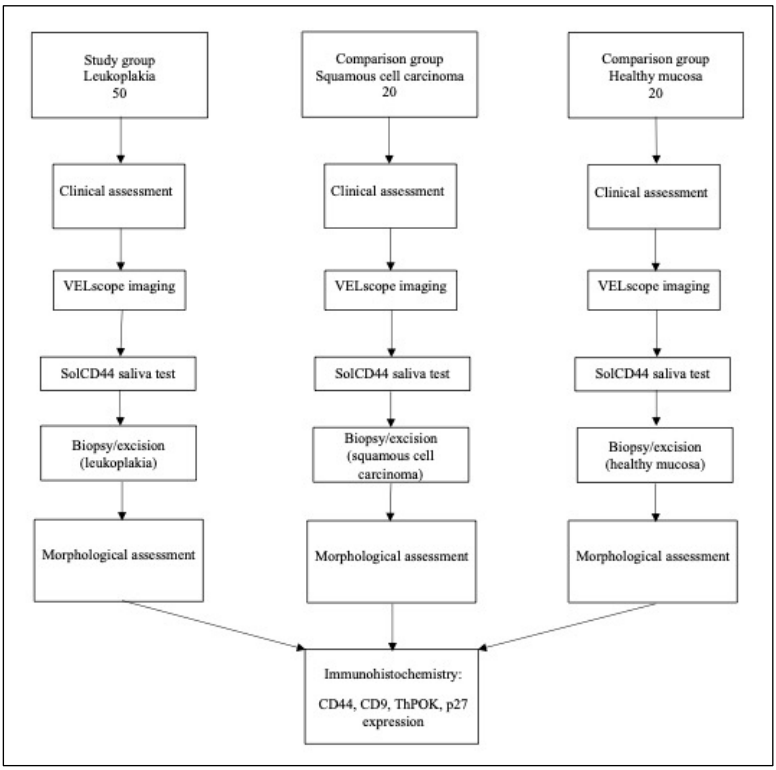


Figure 1.1 **Study design**

1.3 Research inclusion and exclusion criteria

The study comprised one study and two comparison groups, including patients aged 18–85 years. We included 50 patients with suspected OL, subsequently confirmed by clinical and morphological methods; one comparison group consisted of 20 patients with histologically proven

squamous cell carcinoma and the other comparison group consisted of 20 patients with healthy oral mucosa.

Inclusion criteria:

1) patient age 18–85 years;
2) pathological changes in the oral cavity diagnosed for the first time;
3) no previous treatment for malignancy in other organ systems.

Persons under 18 years of age, pregnant women and patients with a history of malignancy in other sites and who had been treated with surgery and/or radiotherapy for oral squamous cell carcinoma were excluded from the study.

1.4 Assessment of healthy, leukoplakia and carcinoma tissue in the oral mucosa with the VELscope fluorescence spectroscopy device

After a thorough extra- and intra-oral examination, patients with oral leukoplakia, squamous cell carcinoma and healthy mucosa were photographed with a Canon Eos 750D camera (Tokyo, Japan) and a Canon EF-S 60 mm f/2.8 macro lens (Figure 2.2). A VELscope device was then attached to the camera with an adapter, allowing the fluorescence of the illuminated tissue to be photographed and assessed. VELscope is based on direct visualisation of the abnormal tissue as a result of fluorescence. By shining blue light on the oral tissues, the fluorescence disappears in the area of the neoplastic changes that are not visible to the eye, indicating the size of the specific pathological process and the borders for biopsy or surgery (Figure 2.4). OL leukoplakias and healthy mucosa were photographed by the author of the Thesis, carcinomas were photographed by Prof. Čēma and Assoc. Prof. Guntars Selga. All the images were analysed by the author of the Thesis.

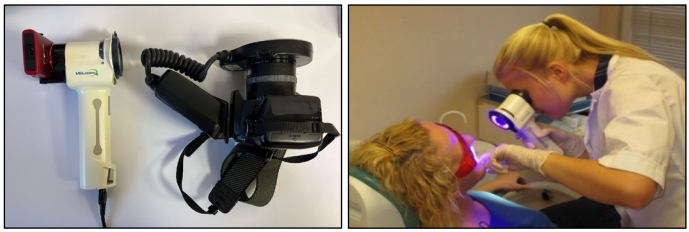


Figure 1.2 **VELscope device and Canon Eos 750D camera**

VELscope examination of the oral cavity. Images by Madara Dzudzilo

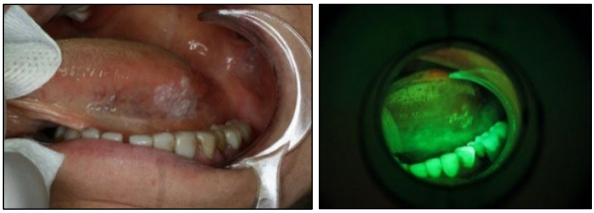


Figure 1.3 **Nodular leukoplakia of the lateral part of the tongue** (left image) **and its visualisation with VELscope** (right image)

Image from the Archive of the Oral Medicine Centre of the Institute of Stomatology

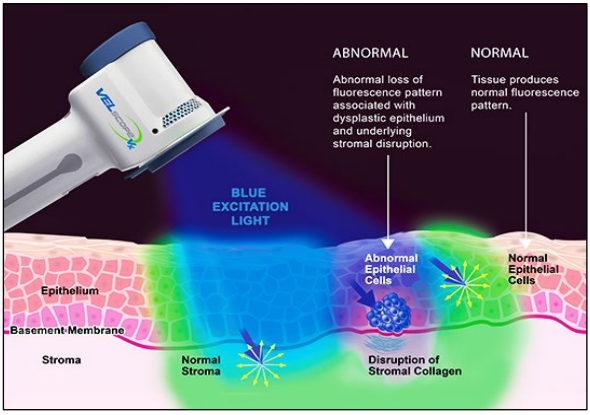


Figure 1.4 **VELscope operating principle**

Image adapted from hewlettdental.com

1.5 Determination of soluble CD44 and total protein in saliva

Soluble CD44 and total protein were assessed using the OncAlert ® Oral Cancer Rapid assay (VIGILANT BIOSCENCES, Ft. Lauderdale, FL, USA) (Figure 1.5). The test device consists of two test cassettes, one with CD44 control and test lines, the other for colorimetric evaluation of total protein. A saliva sample collection container and 5 ml of saline were used to obtain mouth rinses. Mouth rinses were collected before biopsy or excision of the tissue sample. The methodology followed strictly the protocol. The patient was given 5 ml of saline and instructed to rinse the mouth and throat with the solution for 5 seconds and then spit the sample into a container. The test cassette was placed in the mouthwash container for three seconds, then removed and placed on a flat surface for 10 minutes. After 10 minutes, the test results were read. If the test control line did not appear, the test was considered invalid. If the CD44 line appeared, the test was positive. Total protein results were assessed on a colour intensity scale from 0 to 4. As the amount of protein increases, the yellow area changes to green and then to a dark greenish or bluish-green area (Figure 1.6). SolCD44 and TP expression was observed by the author of the Tesis following officially recommended colour gradient scale.

Figure 1.5 **OncAlert Saliva Test**

Image adapted from Franzmann et al., 2018

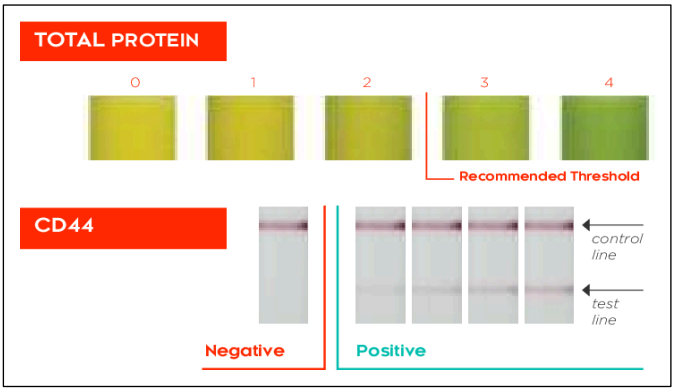


Figure 1.6 **Total protein colour gradient scale**

Image adapted from Franzmann et al., 2018

1.6 Morphological evaluation of healthy oral mucosa, oral leukoplakia and squamous cell carcinoma

Tissue samples obtained during clinical examination were fixed in 10 % neutral buffered formalin solution and sent to the Institute of Pathology, PSCUH. In a further process, the samples were processed in a Sakura Tissue-Tek VIP 5 TM vacuum tissue infiltration processor and embedded in paraplast (Diapath, Bergamo, Italy). Samples from the obtained paraffin blocks were cut into 4 µm thick sections, then placed on adhesive positively charged slides. This was followed by deparaffinisation and rehydration with alcohol to water.

The micropreparations were stained with haematoxylin and eosin and then assessed under a light microscope by two independent morphologists (Prof. Regīna Kleina and dr. Madara Dzudzilo).

The tissue samples of the control group were visually and microscopically unchanged, with the classical structure of the oral mucosa.

The epithelial membrane, basement membrane and submucosa were analysed morphologically. Other diseases of the oral mucosa were excluded. In larger tissue samples, we diagnosed small salivary glands and their ducts.

The following parameters were assessed in oral leukoplakia: size, thickness, its surface, histological type, degree of dysplasia: mild, moderate and severe (according to the 2017 WHO classification) (El-Naggar et al., 2017), distribution of keratinisation processes and stromal cell reaction under the leukoplakia, consisting of plasma cells, lymphocytes and macrophages (Odell et al., 2021).

We analysed oral squamous cell carcinoma specimens stained with haematoxylin for their degree of overgrowth, the expression of epithelial cell atypia to determine the grade (G) of squamous cell carcinoma differentiation, and the sites of invasion through the basement membrane to invasion into submucosal structures. The carcinomas analysed were grouped according to the 2017 WHO classification (El-Naggar et al., 2017), taking into account all information on TMN data available to us.

1.7 Immunohistochemical visualisation and evaluation

Immunohistochemical visualisation of the antigens of interest was performed in formalin-fixed, paraffin-embedded oral leukoplakia, healthy and squamous cell carcinoma tissues treated with primary antibodies (Table 1.1).

Table 1.1

Characteristics of primary antibodies used in study

Antibody	Clone	Manufacturer	Dilution
CD44	DF1485	DAKO	1:50
CD9	60232-1-Ig-20	Proteintech	1:1000
p27	SX53G8	DAKO	1:50
ThPOK	615819	R&D Systems	0.5 µg/mL

CD44, p27, ThPOK and CD9 proteins were assessed by the standard polymer-based visualisation method En-vision. Tissue samples were incubated with 3 % H2O2 for 10 minutes to inhibit endogenous peroxidase activity. Antigen release was performed in freshly prepared 0.01 mol/l sodium citrate buffer at 750 W in a frame of three cycles of 10 min each. Tissue samples were stained with Meyer's haematoxylin. Antigen expression in oral mucosal epithelium and mononuclear cells under the basement membrane was considered positive if more than 10 % of the cells expressed the marker. CD44, CD9, ThPOK and p27 antigen expression was assessed in 3 fields of view at 400 × magnification (Table 1.2). In healthy mucosal tissues and homogeneous leukoplakia, CD44, CD9 antigen expression stained brown in the oral epithelial and stromal cell membrane. In non-homogeneous OL and carcinomas, we assessed the presence of biomarkers in the cytoplasm of the epithelium.

The number of epithelial layers and mononuclear cells expressing CD44 and CD9 antigen were counted. In classical cases, ThPOK and p27 antigen were demonstrated in epithelial nuclei. In contrast, in a proportion of non-homogeneous leukoplakia and carcinomas, we diagnosed p27 expression in the cytoplasm of the squamous epithelium. Photomicrographs of the micropreparations using immunohistochemical staining techniques were taken with Kappa image-based software using an Axiolab microscope (Zeiss, Oberkochen, Germany).

Table 1.2

Characteristics of interpretation of a positive immunohistochemical reaction

Antibody	Antigen expression localisation	Positive reaction interpretation
CD44	Cytolemma	Number of positive epithelial layers per field of view, magnification 400 ×
CD9	Cytolemma	Number of positive epithelial layers per field of view, magnification 400 ×
p27	Nuclei	Number of positive epithelial cell nuclei per field of view, magnification 400 ×

Table 1.2 continued

Antibody	Antigen expression localisation	Positive reaction interpretation
ThPOK	Nuclei	Number of positive epithelial cell nuclei per field of view, magnification 400 ×

1.8 Statistical data analysis

Statistical data analysis was performed using GraphPadPrism 9.0 for MacOS (GraphPad Software, San Diego, CA, USA) to evaluate the results.

The D'Agostino-Pearson, Anderson-Darling and Shapiro-Wilk normality tests were used to check whether the numerical data obtained were normally distributed. Comparisons between groups were made by unpaired t-test or one-way ANOVA or repeated measures two-way ANOVA followed by two-way Benjamini, Krieger and Yekutieli method. Spearman's rank correlation test was used to measure the strength and direction of associations between variables. For this analysis, the categorical data were coded as follows: sex (female, male) was coded as 1 and 2; total protein (TP) levels in 4 grades (1, 2, 3, 4), assuming that 1 and 2 indicate low levels, 3 and 4 indicate high levels; the clinical forms of leukoplakia were divided into three groups, categorised as 1 – homogeneous, 2 – verrucous and nodular and 3 – erythroleukoplakia; the localisation of leukoplakia was characterised by 3 regions: the cheek and lip mucosa (1), the tongue and gums (2), the floor of the mouth (3). For all statistical tests, p values < 0.05 were considered significant. In the Thesis, p values up to 0.0001 are represented in exact numbers. An alluvial diagram was created using Jamovi program to represent the studied parameters. All graphical images and statistical analyses were performed using GraphPadPrism 9.0 software for macOS (GraphPad Software, San Diego, CA, USA). Results are shown as median with interquartile range.

2 Results

2.1 Clinical and morphological characteristics of oral leukoplakia

The study analysed data from 50 patients, 29 (58 %) of whom were male and 21 (42 %) – female (Table 2.1). The minimum age was 27 years, the maximum 82 years; the age range was 55 years. The mean age of patients was M = 57 years; SD = 14.14 years; the modal or most common age was 55 years; the median age was 59 years.

Oral leukoplakia in our study group were located in the buccal mucosa (*mucosae buccae*, n = 18), lateral and ventral surface of the tongue (*pars lateralis et ventrale linguae*, n = 17), floor of the mouth (*fundus cavi oris*, n = 11), gingiva (*gingivae*, n = 2) and labial mucosa (*mucosae labii*, n = 2). In the study group, oral leukoplakia were localised to the left side of the oral cavity (n = 22) and to the right side (n = 15), whereas the remaining leukoplakia were localised to the floor of the mouth, more anteriorly, and to the lingual mucosa (n = 13).

Evaluating the sizes of leukoplakias, we divided them into three groups: 1) < 1 cm – small 2) 1–2 cm – medium and 3) > 2 cm – large. OL of small size was present in 18 cases (36 %), medium size was present in 10 patients (20 %), and larger OL than 2 cm were present in 22 patients (44 %) in surgical material and not in biopsies. Multiple oral leukoplakias were present in 6 cases in our study group, accounting for 12 %.

The following clinical variants of leukoplakia were diagnosed: n = 18 (36 %) homogeneous leukoplakia and n = 32 (64 %) non-homogeneous leukoplakia. Among the non-homogeneous leukoplakias, erythroleukoplakia (n = 17), verrucous leukoplakia (n = 11) and nodular leukoplakia (n = 4) were identified.

Table 2.1

Characteristics of leukoplakia patients

Patient	Age	Gender	Localisation	OL clinical type	CD44 positive epithelial layers in OL, mean (40 × 10)	CD9 positive epithelial layers in OL, mean (40 × 10)	p27 positive epithelial nuclei in OL, mean (40 × 10)	Makrophages, mean, in one vie field (40 × 10)	SolCD44, reaction positive/negati	Total protein (TP) color intensity	Dysplasia grade in OL
1 - R	68	F	*Fundus cavi oris*	Homo-geneous	15	16	82	23	–	high 3	Hyperplasia w/o dysplasia
2 - K	65	F	*p. lateralis linguae sin*	Verrucous	15	13	47	29	–	high 3	Hyperplasia w/o dysplasia
3 - P	59	F	*p. lateralis linguae sin*	Nodular	15	28	94	14	–	high 3	Mild dysplasia
4 - P	68	M	*p. lateralis linguae dx*	Verrucous	16	17	134	11	–	high 3	Mild dysplasia
5 - E	27	M	*Muccosa buccae dx*	Erythro-leukoplakia	14	15	88	15	–	high 3	Severe dysplasia
6 - T	56	F	*p. lateralis linguae dx*	Erythro-leukoplakia	15	25	73	29	–	low 2	Hyperplasia w/o dysplasia
7 - M	50	F	*Fundus cavi oris*	Erythro-leukoplakia	18	19	52	23	+	high 4	Severe dysplasia
8 - K	62	M	*Muccosa buccae sin*	Nodular	17	17	81	17	+	high 3	Mild dysplasia
9 - B	40	F	*Muccosa buccae dx*	Erythro-leukoplakia	21	20	69	23	+	low 2	Mild dysplasia
10 - P	59	F	*Muccosa buccae sin*	Homo-geneous	11	16	117	28	+	low 2	Hyperplasia w/o dysplasia

Table 2.1 continued

Patient	Age	Gender	Localisation	OL clinical type	CD44 positive epithelial layers in OL, mean (40 × 10)	CD9 positive epithelial layers in OL, mean (40 × 10)	p27 positive epithelial nuclei in OL, mean (40 × 10)	Makrophages, mean, in one vie field (40 × 10)	SolCD44, reaction positive/negati	Total protein (TP) color intensity	Dysplasia grade in OL
11 - L	78	F	*Fundus cavi oris*	Erythro-leukoplakia	11	18	76	21	+	high 4	Severe dysplasia
12 - D	62	F	*Muccosa buccae sin*	Erythro-leukoplakia	16	16	58	24	+	high 3	Moderate dysplasia
13 - O	55	M	*Muccosa buccae sin*	Verrucous	15	16	56	23	+	low 2	Hyperplasia w/o dysplasia
14 – V	65	F	*Muccosa buccae dx*	Erythro-leukoplakia	18	19	75	22	+	low 2	Hyperplasia w/o dysplasia
15 - P	38	M	*Fundus cavi oris*	Erythro-leukoplakia	13	15	65	23	+	high 3	Severe dysplasia
16 - S	63	F	*Muccosa buccae sin*	Nodular	16	16	79	25	–	low 2	Hyperplasia w/o dysplasia
17 - P	66	M	*Fundus cavi oris*	Verrucous	19	28	16	22	+	low 1	Hyperplasia w/o dysplasia
18 - M	37	M	*Mucosa proc. alveolaris mandibulae sin*	Homo-geneous	19	21	250	14	+	low 2	Mild dysplasia
19 - G	81	M	*p.lateralis linguae sin*	Erythro-leukoplakia	17	17	115	17	+	high 3	Severe dysplasia
20 - M	56	F	*Muccosa buccae dx*	Homo-geneous	24	27	111	24	–	low 2	Hyperplasia w/o dysplasia

Table 2.1 continued

Patient	Age	Gender	Localisation	OL clinical type	CD44 positive epithelial layers in OL, mean (40 × 10)	CD9 positive epithelial layers in OL, mean (40 × 10)	p27 positive epithelial nuclei in OL, mean (40 × 10)	Makrophages, mean, in one vie field (40 × 10)	SolCD44, reaction positive/negati	Total protein (TP) color intensity	Dysplasia grade in OL
21 - R	41	M	*Fundus cavi oris*	Homo-geneous	15	17	67	28	–	low 2	Hyperplasia w/o dysplasia
22 - S	82	M	*p.lateralis linguae sin*	Verrucous	18	18	157	12	+	high 3	Mild dysplasia
23 - O	79	M	*Muccosa buccae sin*	Erythro-leukoplakia	10	9	120	8	+	high 4	Severe dysplasia
24 -V	64	M	*Muccosa labii inferior*	Verrucous	10	10	302	5	+	high 3	Moderate dysplasia
25 - J	29	M	*Muccosa buccae dx*	Verrucous	14	24	98	19	+	high 3	Moderate dysplasia
26 - S	34	M	*Muccosa buccae dx*	Homo-geneous	18	20	187	17	–	low 2	Mild dysplasia
27 - F	70	F	*Mucosa proc. alveolaris mandibulae dx*	Homo-geneous	19	20	137	27	+	low 2	Hyperplasia w/o dysplasia
28 - K	39	M	*p.lateralis linguae sin*	Erythro-leukoplakia	13	16	182	23	+	high 3	Severe dysplasia
29 - N	41	M	*p.lateralis linguaes sin*	Erythro-leukoplakia	22	24	87	17	–	low 2	Severe dysplasia
30 - P	55	M	*Muccosa buccae dx*	Verrucous	14	18	103	19	+	high 3	Moderate dysplasia

Table 2.1 continued

Patient	Age	Gender	Localisation	OL clinical type	CD44 positive epithelial layers in OL, mean (40 × 10)	CD9 positive epithelial layers in OL, mean (40 × 10)	p27 positive epithelial nuclei in OL, mean (40 × 10)	Makrophages, mean, in one vie field (40 × 10)	SolCD44, reaction positive/negati	Total protein (TP) color intensity	Dysplasia grade in OL
31 - L	78	M	*p.lateralis linguae sin*	Erythro-leukoplakia	25	25	162	24	+	high 3	Severe dysplasia
32 - A	63	F	*p.lateralis linguae sin*	Erythro-leukoplakia	17	18	39	11	+	high 3	Severe dysplasia
33 - S	51	M	*Muccosa buccae sin*	Homo-geneous	22	24	167	14	–	low 2	Mild dysplasia
34 - S	65	F	*Fundus cavi oris*	Erythro-leukoplakia	16	16	32	11	+	high 4	Severe dysplasia
35 - M	56	M	*p.lateralis linguae sin*	Verrucous	12	13	98	12	+	high 3	Moderate dysplasia
36 - S	64	M	*Muccosa buccae sin*	Homo-geneous	16	17	99	19	+	high 3	Hyperplasia w/o dysplasia
37 - S	29	F	*p.lateralis linguae sin*	Homo-geneous	24	25	199	22	-	low 2	Hyperplasia w/o dysplasia
38 - M	62	M	*Fundus cavi oris*	Verrucous	12	10	101	31	+	high 3	Moderate dysplasia
39 - C	69	F	*Fundus cavi oris*	Erythro-leukoplakia	8	8	30	9	+	high 4	Severe dysplasia
40 - D	58	F	*Muccosa buccae dx*	Homo-geneous	19	20	239	27	–	low 2	Hyperplasia w/o dysplasia

Table 2.1 continued

Patient	Age	Gender	Localisation	OL clinical type	CD44 positive epithelial layers in OL, mean (40 × 10)	CD9 positive epithelial layers in OL, mean (40 × 10)	p27 positive epithelial nuclei in OL, mean (40 × 10)	Makrophages, mean, in one vie field (40 × 10)	SolCD44, reaction positive/negati	Total protein (TP) color intensity	Dysplasia grade in OL
41 - D	42	F	*Muccosa labii inferior*	Homo-geneous	15	16	180	28	–	low 2	Hyperplasia w/o dysplasia
42 - K	43	M	*p.lateralis linguae sin*	Verrucous	10	12	74	12	+	high 4	Moderate dysplasia
43 - K	49	F	*p.lateralis linguae dx*	Homo-geneous	22	23	165	14	+	low 2	Mild dysplasia
44 - O	63	M	*Muccosa buccae sin*	Homo-geneous	18	19	187	13	–	low 2	Mild dysplasia
45 - D	67	M	*Fundus cavi oris*	Homo-geneous	22	22	123	18	–	low 2	Mild dysplasia
46 - R	66	F	*p.lateralis linguae dx*	Homo-geneous	19	20	207	15	–	low 2	Mild dysplasia
47 - B	49	M	*Fundus cavi oris*	Erythro-leukoplakia	11	10	17	6	+	high 4	Severe dysplasia
48 - J	77	M	*p.lateralis linguae dx*	Homo-geneous	24	25	377	24	–	low 2	Hyperplasia w/o dysplasia
49 - M	55	M	*p.lateralis linguae sin*	Nodular	14	15	88	23	+	high 3	Moderate dysplasia
50 - L	55	M	*Muccosa buccae dx*	Homo-geneous	22	23	256	21	–	low 2	Hyperplasia w/o dysplasia

2.2 Clinical and morphological characteristics of oral squamous cell carcinoma

Data from 22 patients were analysed, but we excluded 2 cases because the clinician's diagnosis of carcinoma was not confirmed, and they were morphologically oral leukoplakia. The minimum age was 43 years, the maximum – 73 years; and the age range was 30 years. The mean age of women was M = 63.5 years; SD = 14.14 years; the modal or most common age was 62 years; the median age was 55 years. In contrast, men were younger with a mean age of 58.8 years, SD = 12.11 years; the modal or most common age was 55 years; the median age was 59 years. 12 (60 %) of the patients were male and 8 (40 %) were female.

The clinical (macroscopic) presentation of oral carcinomas was predominantly ulcerations (n = 17), with erythroplakia in 3 cases. The grades of carcinoma differentiation were as follows: Grade 1 and 3 each in 25 % of cases, and moderately differentiated carcinomas – Grade 2 – in 50 % of oncological patients analysed. In some cases, background carcinomas were also found: papilloma with severe dysplasia and transformation into carcinoma (n = 2), malignisation of leukoplakia (n = 2) and chronic sialodenitis with malignisation at its outlet end (n = 1).

Carcinomas in our study group were localised in the floor of the mouth (n = 8, 40 %), in the tongue and gingival mucosa equally – in 20 % of cases in each site (n = 4). Carcinomas of other sites (buccal mucosa, etc.) accounted for 20 % (n = 4).

The distribution of oral carcinomas by clinical stage was as follows:

1) T_o (n = 2 with N_oM_o);

2) T_1 (n = 2 with T_1 N_oM_o);

3) T_2 (n = 9 with T_2 N_oM_o);

4) T_3 (n = 2 with T_2 N_1M_o);

5) T_{4A} (n = 3 with T_4 N_oM_o);

T_{4B} (n = 2 with T_4 N_1M_o)

2.3 VELscope imaging characteristics in patients with healthy mucosa, leukoplakia and squamous cell carcinoma

For tissue fluorescence assessment and biopsy site identification, 20 patients with healthy oral mucosa, 50 patients with oral leukoplakia and 20 patients with oral squamous cell carcinoma were examined with the VELscope device.

2.3.1 Healthy oral mucosal tissue

In normal oral tissues, epithelial autofluorescence is induced by basal and str. intermedium cells, while stromal fluorescence is caused by structural fibres. When viewed through the VELscope system, healthy oral tissues fluoresced green. A slight loss of fluorescence could be observed in areas with local inflammation. Although a physiological loss of fluorescence indicates epithelial changes, it could also be observed in hyperaemia, hyperkeratosis and other benign changes. In some cases, fluorescence disappeared in the dorsal part of the tongue where bacteria colonised on hypertrophic keratinised – horny thread-like papillae, fluorescing bright orange-red (Figure 2.1). This is called porphyrin fluorescence, which is caused by porphyrins secreted by the microorganisms.

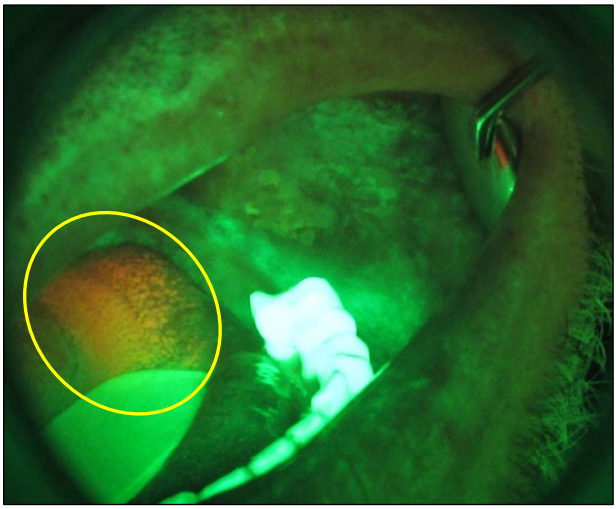


Figure 2.1 **Oral tissue visualisation with the VELscope device**

The area marked with a yellow circle indicates fluorescence in bright orange colour under the influence of bacteria. Image from the Archive of the Oral Medicine Centre of the Institute of Stomatology

2.3.2 Homogeneous and non-homogeneous leukoplakia

Evaluation of clinical images of oral leukoplakia under artificial and VELscope light showed a marked loss of tissue fluorescence in areas with erosive changes. Altered oral mucosa appears as a greyish dark irregularly shaped area on fluorescence.

Homogeneous leukoplakia is depicted by the VELscope as a grey-black patch of uneven intensity with a defined border (Figure 2.2). Adjacent healthy tissue fluoresces light green.

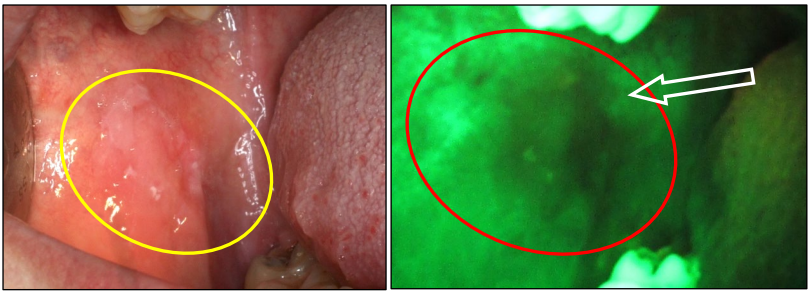


Figure 2.2 **Imaging of homogeneous leukoplakia in buccal mucosa under artificial light** (left) **and VELscope** (right)

The arrow indicates the most pronounced loss of fluorescence.
Image from the Archive of the Oral Medicine Centre of the Institute of Stomatology

Under VELscope light, non-homogeneous leukoplakia visualised dark grey tissue that had lost fluorescence. Compared to homogeneous leukoplakia, the areas of altered tissue were dark grey-black. However, the highest colour intensity was observed in lesions with erosive changes (Figure 2.3). This may be due to increased hyperaemia and small haemorrhages. Clinically, the oral leukoplakia were less visible than when viewed with the VELscope device. However, in some cases, the clinically visible leukoplakia were extensive and the colour intensity demonstrated by the VELscope was variable, ranging from a slight loss of fluorescence to dark grey (Figure 2.4).

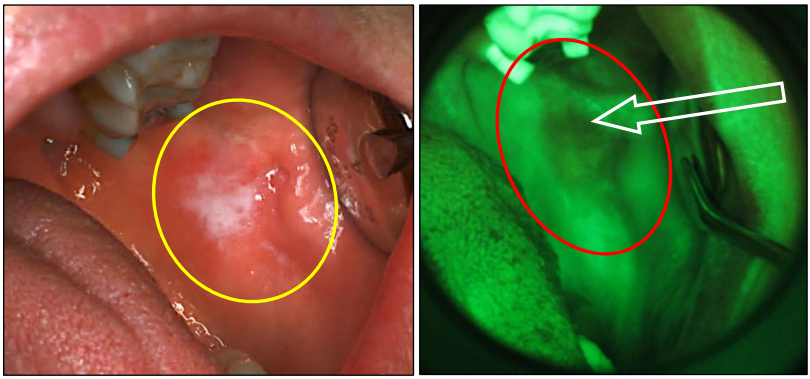


Figure 2.3 **Imaging of erosive leukoplakia in buccal mucosa under artificial light** (left) **and VELscope** (right)

The arrow indicates the most pronounced loss of fluorescence.
Image from the Archive of the Oral Medicine Centre of the Institute of Stomatology

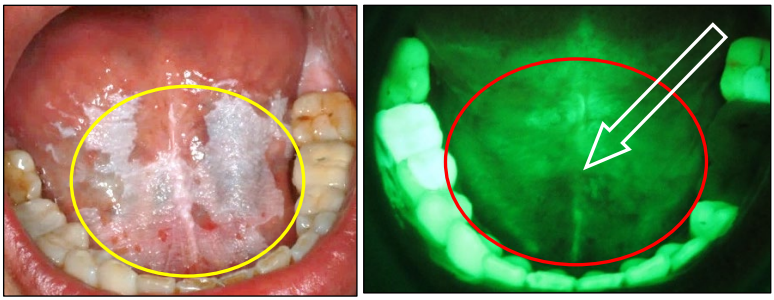


Figure 2.4 **Visualisation of verrucous leukoplakia in the mucosa of the floor of the mouth and ventral surface of the tongue under artificial light** (left) **and VELscope** (right)

The arrow indicates the most pronounced loss of fluorescence.
From the Archive of the Oral Medicine Centre of the Institute of Stomatology

2.3.3 Oral squamous cell carcinomas

In patients with oral carcinoma (Figures 2.5 and 2.6), the areas illuminated by the VELscope appeared as a dark grey area and its borders were much wider and irregular than visually visible, improving the identification of the surgical site.

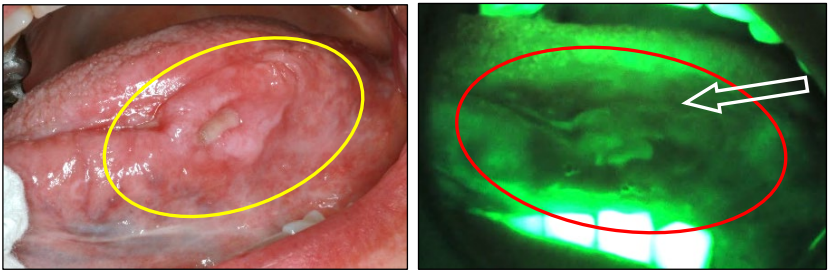


Figure 2.5 **Imaging of oral squamous cell carcinoma** (on the background of nodular leukoplakia) **in the mucosa of the left side of the tongue under artificial light** (left) **and VELscope** (right)

The arrow indicates the most pronounced loss of fluorescence.
Image from the Archive of the Oral Medicine Centre of the Institute of Stomatology

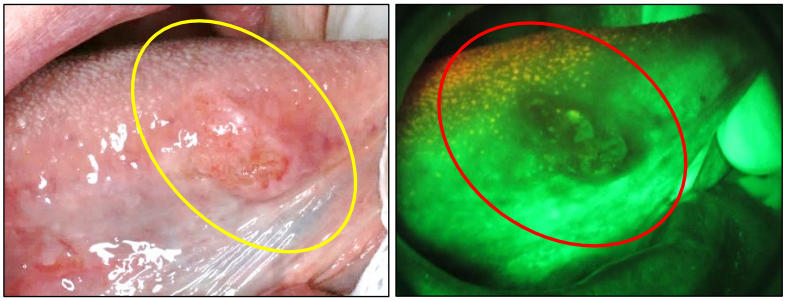


Figure 2.6 **Imaging of oral squamous cell carcinoma in the mucosa of the left side of the tongue under artificial light** (left) **and VELscope** (right)

Image from the Archive of the Oral Medicine Centre of the Institute of Stomatology

2.4 Characterisation of salivary soluble CD44 and total protein in patients with healthy mucosa, leukoplakia and squamous cell carcinoma and comparison with tissue CD44 protein expression

2.4.1 Healthy oral mucosal tissue

In patients with normal oral mucosa, the OncAlert Oral Cancer Rapid test was 100 % negative. Total protein analysis showed colour intensity 1 in 12 patients and colour intensity 2 in 8.

2.4.2 Homogeneous and non-homogeneous leukoplakias

The OncAlert Oral Cancer Rapid test showed that only 5 patients with homogeneous leukoplakia (n = 18) had a positive soluble CD44 test line, while 24 of 32 patients with non-homogeneous leukoplakia had a positive test line.

Total protein results in homogeneous leukoplakia patients: colour intensity 2 was detected in 16 patients, colour intensity 3 appeared in 2 patients. As the total protein level increased, the intensity of the test colours increased to a bluish-greenish colour as determined by the colour scale.

Non-homogeneous leukoplakia patients: colour intensity 2 appeared in 7 patients, colour intensity3 was detected in 18 patients and colour intensity was verified in 7 patients, indicating an increase in total protein.

Using Spearman's rank correlation matrix (Figure 2.7), the data of the study group were analysed. The amount of positive soluble CD44 tests was greater than the total protein intensity value (TP) ($p = 0.0005$), and increased with more severe clinical form of oral leukoplakia ($p = 0.0006$). The number of positive soluble CD44 tests also increased when the CD44-expressing epithelial layers decreased ($p = 0.0086$). A statistically significant difference was demonstrated between the amount of total protein based on the colour intensity scale and the clinical form of oral leukoplakia; the higher the protein level, the more severe the clinical form of leukoplakia ($p < 0.0001$).

A statistically significant negative correlation was found between higher total protein levels and reduced positive CD44 immunohistochemical expression in leukoplakia tissue ($p < 0.0001$). No correlation was found between leukoplakia localisation and other parameters investigated.

We observed a trend between a higher number of CD44-stained macrophages under oral leukoplakia and lower total protein levels, which was manifested as lower colour intensity on the colour scale ($p = 0.0043$) (Figure 2.7).

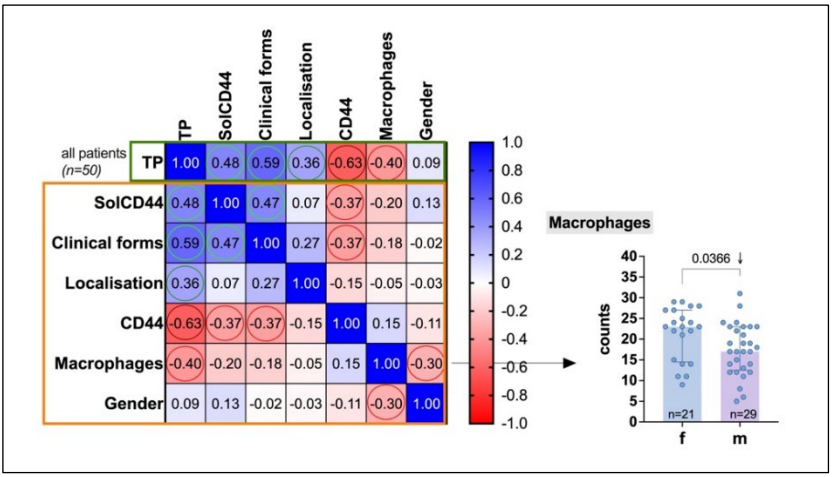


Figure 2.7 **Strength of association of the studied variables**

Soluble CD44, clinical types of leukoplakia, localisation, mean number of CD44-labeled epithelial layers expressed in epithelial layers in oral leukoplakia, mononuclear cells (macrophages under the basement membrane) and gender of leukoplakia patients (n = 50)) with respect to total protein levels (and covariates). Spearman's rank correlation matrix: numbers in squares show the value of the correlation coefficient, which shows the strength of the association; coloured circles represent the most significant associations (red – negative; green – positive). Bar chart: s – women, v – men; the decimal number indicates the p value

Evaluating the female group separately (Figure 2.8), a statistically significant correlation ($p < 0.0001$) was found between the localisation of

leukoplakia (pars lateralis linguae) and a higher level of total protein (colour intensity 3 or 4).

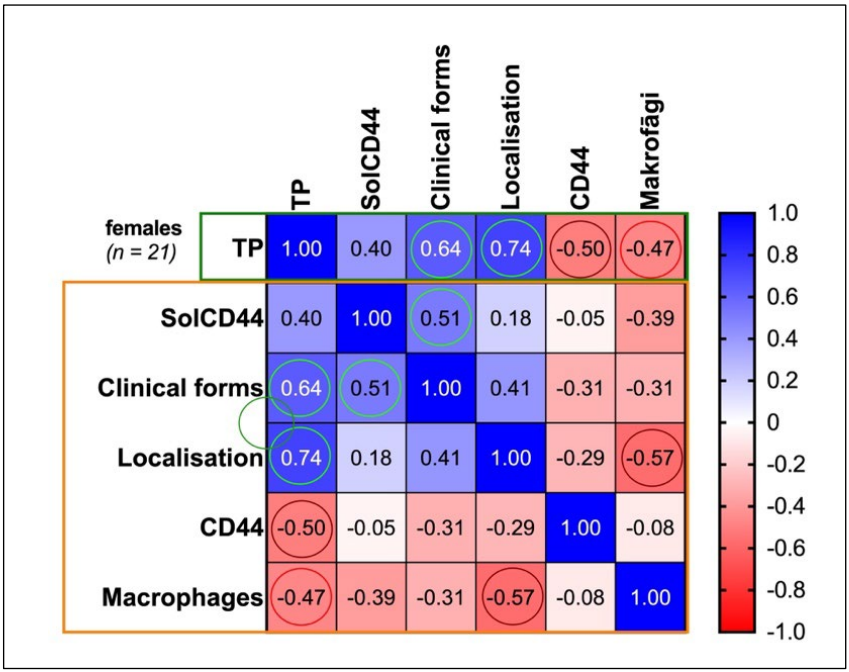


Figure 2.8 **Spearman's rank correlation matrix for the female group**

The strength of association of the variables studied: soluble CD44, clinical form of leukoplakia, location of oral leukoplakia, mean number of CD44-labelled epithelial layers expressed in leukoplakia tissue, mononuclear cells (macrophages under the basement membrane) and sex of leukoplakia patients (n = 21)) with total protein levels (and covariates) in the female group. Spearman rank correlation matrix: numbers in squares show the value of the correlation coefficient, which indicates the strength of the association; coloured circles indicate the most significant associations (red for negative; green for positive)

In the male group (Figure 2.9), a statistically significant negative correlation was observed when total protein was higher but the number of CD44-expressing epithelial layers in leukoplakia was reduced ($p < 0.0001$).

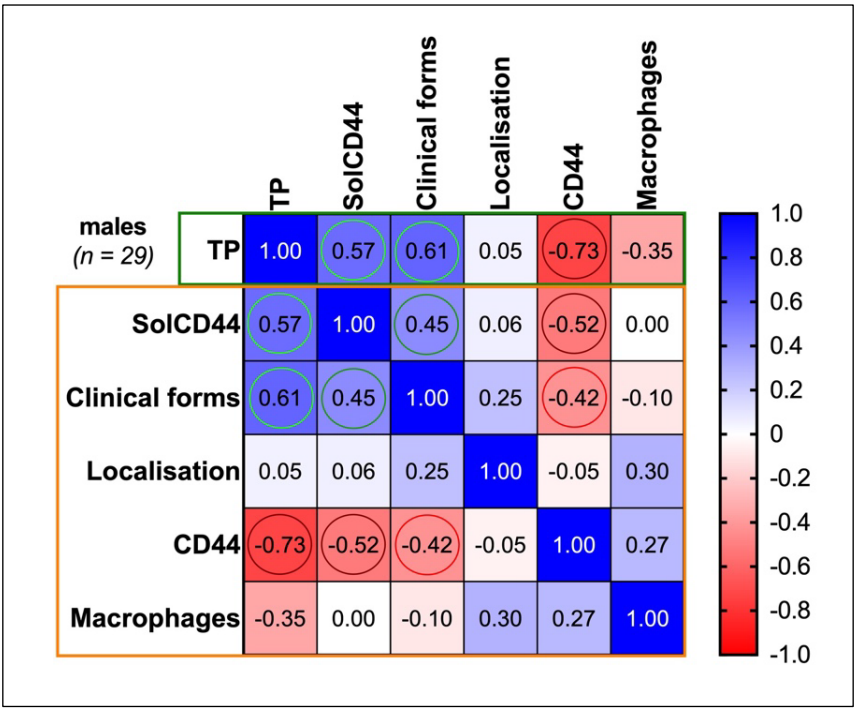


Figure 2.9 **Spearman's rank correlation matrix for the male group**

The strength of association of the variables studied: soluble CD44, clinical form of leukoplakia, location of oral leukoplakia, mean number of CD44-labelled epithelial layers expressed in leukoplakia tissue, mononuclear cells (macrophages under the basement membrane) and sex of leukoplakia patients (n = 21)) with total protein levels (and covariates) in the female group. Spearman rank correlation matrix: numbers in squares show the value of the correlation coefficient, which indicates the strength of the association; coloured circles indicate the most significant associations (red for negative; green for positive)

Figure 2.10 illustrates the paired correlation between total protein, soluble CD44, mean number of CD44-expressing epithelial layers in leukoplakia tissue and number of macrophages in connective tissue between the control group and patients with oral leukoplakia. $p < 0.0001$ indicates a statistically significant difference between the groups.

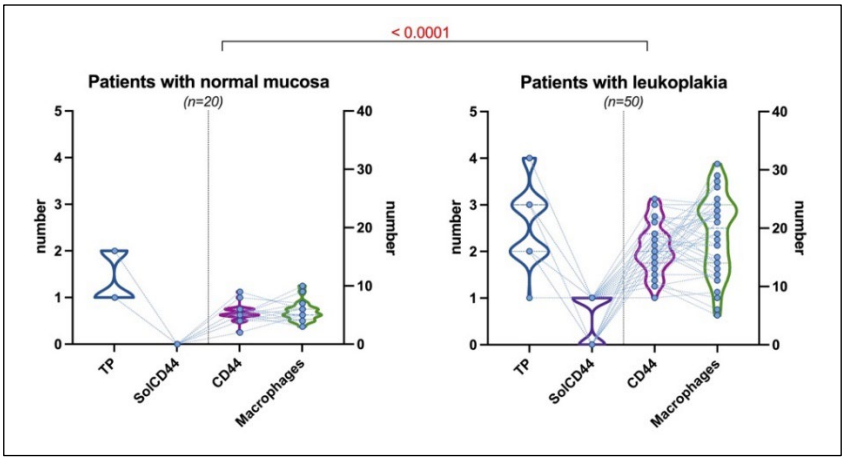


Figure 2.10 **Paired size comparison between patients with healthy mucosa and leukoplakia**

Statistical significance (*p*, decimal numbers) was determined by group ANOVA test

The diagram (Figure 2.11) demonstrates the correlation of the different clinical forms of leukoplakia included in our study with the degree of oral leukoplakia dysplasia, total protein colour intensity, sex and soluble CD44 (negative – 0, positive – 1). Homogeneous leukoplakia (pink ribbon) has the highest correlation with hyperplasia and mild dysplasia, total protein colour intensity 2 and negative soluble CD44 test. Nodular and verrucous leukoplakia (green ribbon) most strongly associated with mild to moderate dysplasia, total protein colour intensity 3 and positive soluble CD44 test. Erythroleukoplakia (blue ribbon) has the highest correlation with severe dysplasia and Ca *in situ*, total protein colour intensity 3 and 4 and a positive soluble CD44 test.

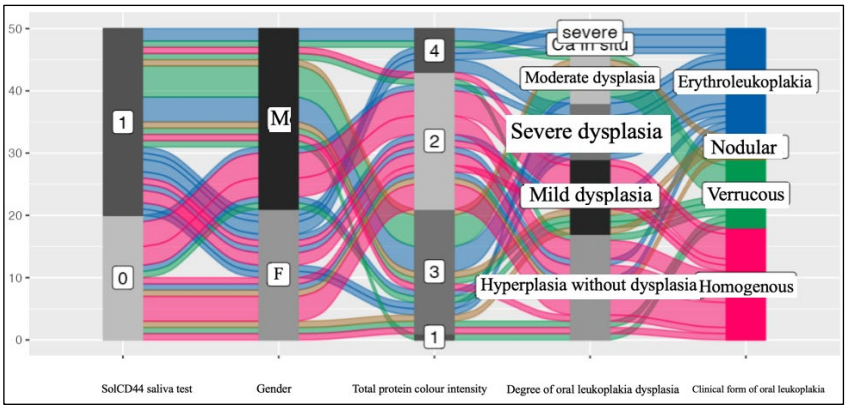


Figure 2.11 **Representation of the studied parameters of oral leukoplakia in the alluvial diagram**

Parameters compared in the diagram: soluble CD44 test (1 – positive, 0 – negative), gender (F – female, M – male), total protein colour intensity (1–4), degree of oral leukoplakia dysplasia (hyperplasia without dysplasia, mild dysplasia, moderate dysplasia, severe dysplasia, Ca *in situ*), clinical form of leukoplakia (homogeneous, verrucous, nodular, erythroleukoplakia)

2.4.3 Oral squamous cell carcinomas

In patients with oral mucosa squamous cell carcinoma, the OncAlert Cancer Rapid test was 100 % positive for soluble CD44. 12 patients had total protein colour intensity 3 and 8 patients had colour intensity 4.

2.5 Immunohistochemical characterisation of CD44 antigen in oral mucosal tissues

2.5.1 Healthy oral mucosal tissue

CD44 antigen expression was assessed in healthy oral mucosal tissues, where CD44-positive oral epithelium was observed in an average of 5 *str. basale* and *str. intermediate* cell layers, but was not expressed in the epithelium of the upper mucosal layers. Positive expression of CD44 protein was restricted to

cell membranes. CD44 glycoprotein was also detected on average in five mononuclear cells under the basement membrane in one field of view of 400 × (Figure 2.12). CD44 expression was compared in three whole mucosal points (two at the edges and one in the centre of the microarray). In normal mucosa, CD44 expression in the epithelial membrane was not statistically significantly different in all three points of the tissue sample.

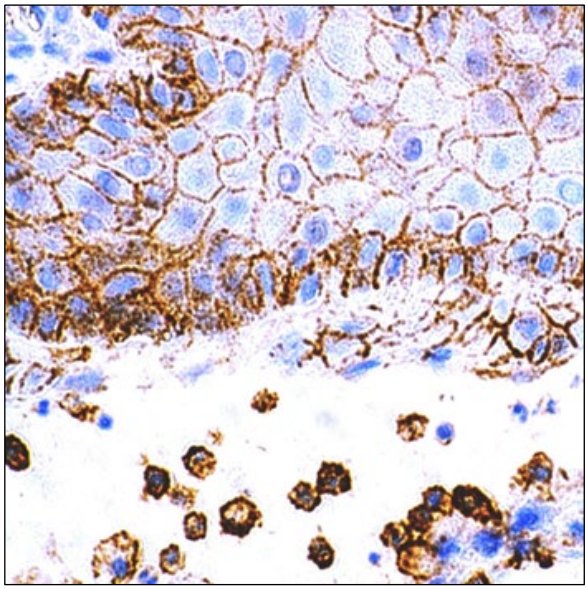


Figure 2.12 **CD44 antigen expression on epithelial membranes and rare mononuclear cells beneath the basement membrane in healthy oral mucosa**

Immunoperoxidase, anti-CD44, 400 × magnification.
Image by Madara Dzudzilo

2.5.2 Homogeneous and non-homogeneous leukoplakias

In homogeneous oral leukoplakia, membranous CD44 glycoprotein was expressed on average in the cell membranes of 19 epithelial layers (Figure 2.13). Antigen expression was statistically significantly higher at the edges of the leukoplakia ($p = 0.0224$) compared with its centre ($p = 0.0019$).

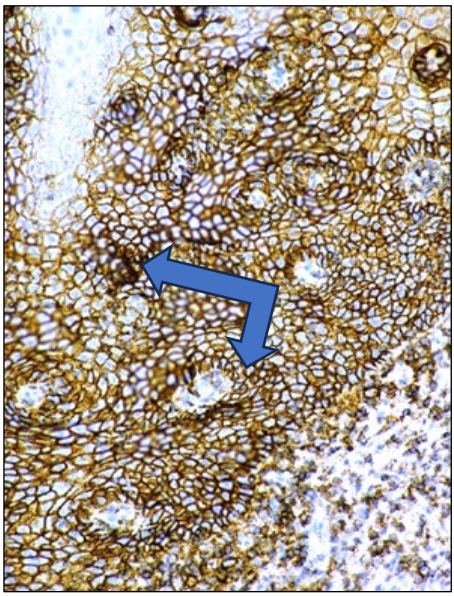


Figure 2.13 **CD44 antigen expression in the increased number of epithelial layers in oral leukoplakia** (arrow)

Immunoperoxidase, anti-CD44,
100 × magnification.
Image by Madara Dzudzilo

In non-homogeneous leukoplakia, CD44 protein was expressed in an average of 15 epithelial layers, and in 47 % of these leukoplakia, CD44 antigen was present not only in the membrane of the epithelial cell but also in the cytoplasm of the leukoplakia epithelium (Figure 2.14).

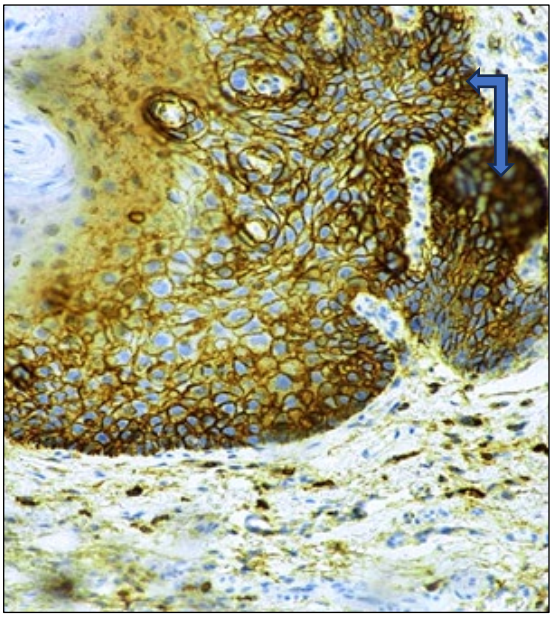


Figure 2.14 **Membranous and intra-cytoplasmic expression of CD44 glycoprotein in non-homogeneous oral leukoplakia** (arrow)

Immunoperoxidase, anti-CD44,
200 × magnification.
Image by Madara Dzudzilo

CD44 antigen expression was also detected in the acinus of small salivary glands and their ducts under the basement membrane. Notably, in dilated ducts with atrophic epithelium, CD44 protein expression is significantly reduced (Figure 2.15).

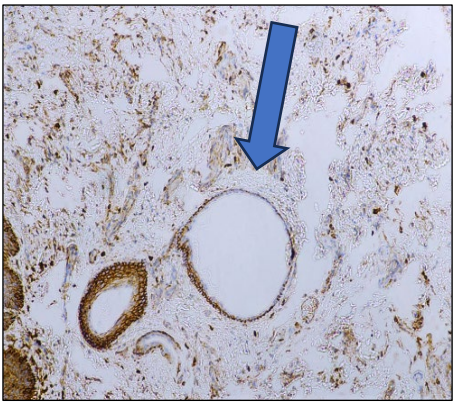


Figure 2.15 **Differential expression of CD44 antigen in the acini and ducts of small salivary glands in the oral submucosa** (arrow)

Immunoperoxidase, anti-CD44,
100 × magnification.
Image by Madara Dzudzilo

CD44 protein was not demonstrated in cell membranes or in the cytoplasm, where keratohyalin granules were present in oral squamous epithelium. Keratohyalin granules are also known to be closely associated with tonofibrils and promote aggregation and cross-linking between cytokeratin filaments of the keratinised layer. In micropreparations of the oral mucosa, they are stained bright blue with haematoxylin. (Figure 2.16).

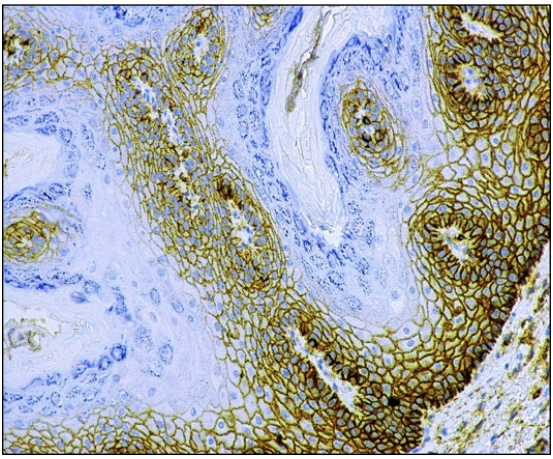


Figure 2.16 **Lack of CD44 antigen expression in squamous epithelial layers with the presence of keratohyalin granules**

Immunoperoxidase, anti-CD44,
200 × magnification.
Image by Madara Dzudzilo

The average number of CD44-labeled mononuclear cells in the OL area under the basement membrane was 18 and the difference was statistically significant ($p < 0.0001$) compared to morphologically unchanged tissue samples ($p < 0.0001$). There was a moderate correlation ($p = 0.0045$) between the number of CD44-positive epithelial layers in the central part of leukoplakia and the marked stromal cells of the *lamina propria* mucosa in this part (Figure 2.17). CD44 antigen was diagnosed to accumulate simultaneously in epithelial membranes and *lamina propria* cells.

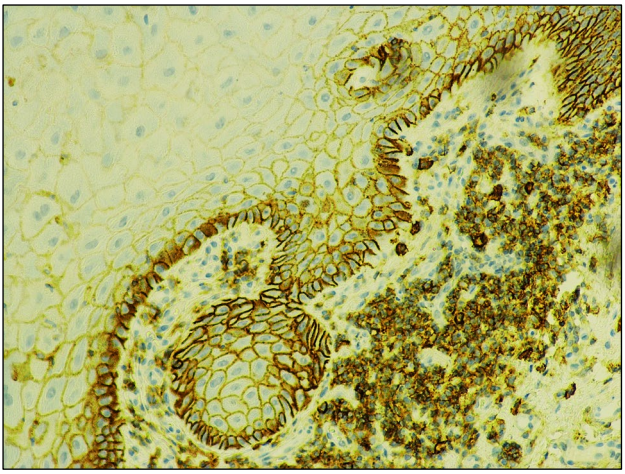


Figure 2.17 **A large number of CD44-positive mononuclear cells in the subbasal area of oral leukoplakia**

Immunoperoxidase, anti-CD44, 400 × magnification.
Image by Madara Dzudzilo

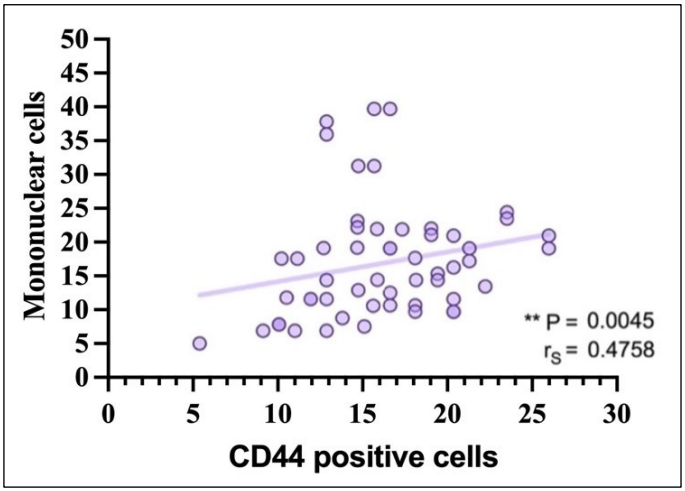


Figure 2.18 **Correlation between the number of CD44-positive epithelial layers and *lamina propria* mucosal mononuclear cells in central part of leukoplakia**

Non-parametric Spearman's test: rS – Spearman's correlation coefficient

Comparing the clinical form of oral leukoplakia with the average number of epithelial layers expressing the CD44 antigen in them, a weak correlation between these parameters was demonstrated (Figure 2.19).

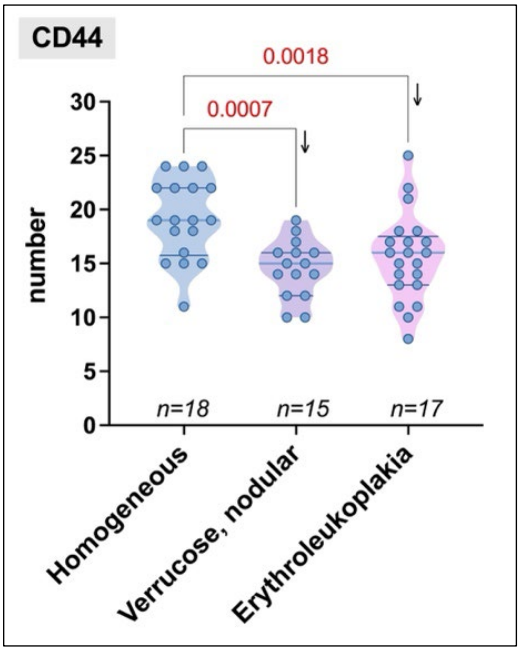


Figure 2.19 **Comparison of the mean number of CD44-labeled epithelial layers between different types of oral leukoplakia**

Statistical significance (*p*, decimal numbers) was determined by ANOVA followed by a post-test (see Materials and Methods section)

2.5.3 Oral squamous cell carcinomas

20 carcinoma cases of the comparison group were analysed. Carcinomas with CD44 expression above 10 % in the central area and invasion site were considered positively labelled samples. The presence of CD44 antigen was diagnosed in all squamous cell carcinomas, but its amount was highly variable, from 10 to 80 % of all malignant cells. The pattern of CD44 expression in squamous cell carcinoma tissue was focal (Figure 2.20).

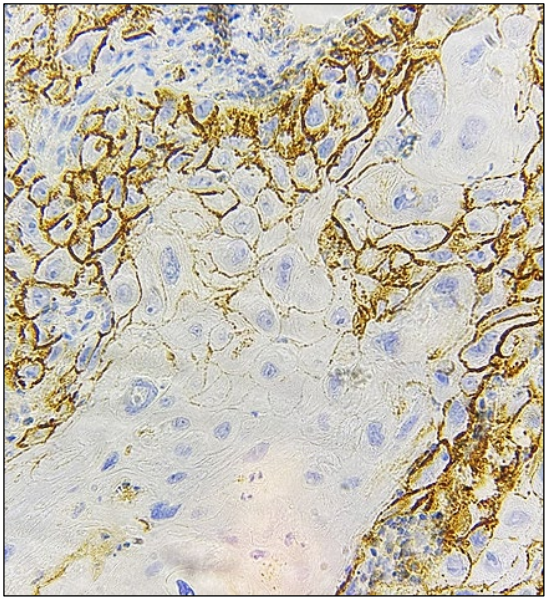


Figure 2.20 **Membranous expression of CD44 antigen in the periphery of carcinoma cell groups, but absent in the middle**

Immunoperoxidase, anti-CD44,
400 × magnification.
Image by Madara Dzudzilo

In highly differentiated carcinomas (G1 and G2), CD44 labelling was strongly membranous, and groups of carcinoma cells without CD44 antigen expression, including at epithelial overgrowth sites, were diagnosed, whereas in poorly differentiated carcinomas (G3) CD44 glycoprotein was detected both in carcinoma cell membranes and in the cytoplasm of malignant cells (Figures 2.21 and 2.22).

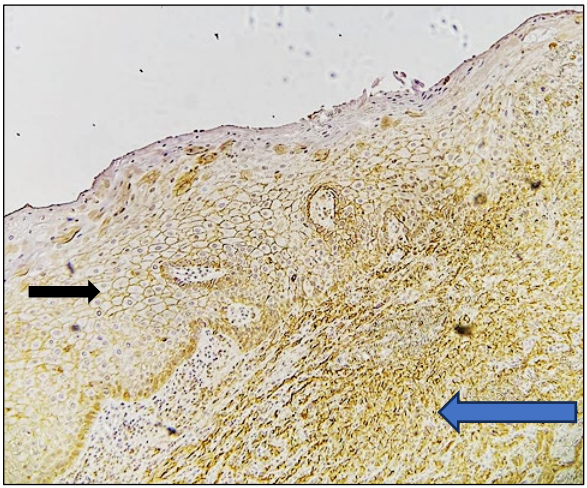


Figure 2.21 **Membranous expression of CD44 glycoprotein in hyperplastic oral mucosal epithelium overlying carcinoma** (black arrow) **but intracytoplasmic expression in malignant cells invading carcinoma below basement membrane** (blue arrow)

Immunoperoxidase, anti-CD44, 100 × magnification.
Image by Madara Dzudzilo

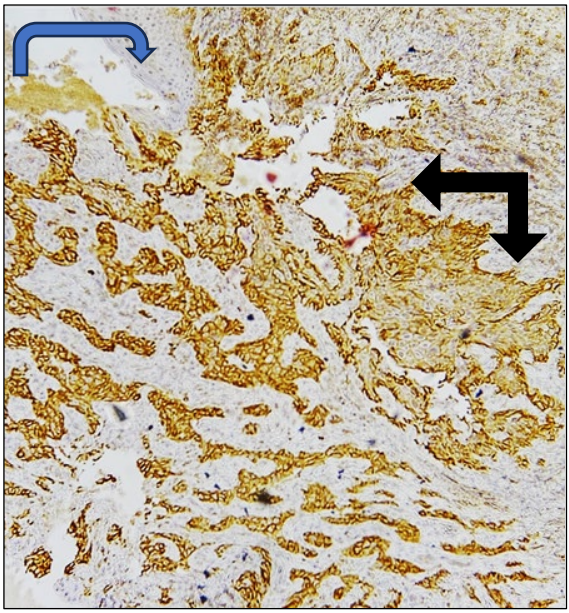


Figure 2.22 **Microscopic ulceration** (blue arrow) **on the surface of a poorly differentiated oral carcinoma** (blue arrow) **with intracytoplasmic CD44 expression in malignant cells** (black arrow)

Immunoperoxidase, anti-CD44, 100 × magnification.
Image by Madara Dzudzilo

The carcinomas we analysed had 200 or more disorganised layers, a number influenced by the T stage of the carcinoma and especially by the depth of tumour invasion into the submucosa of the oral cavity. However, the mean number of CD44-labelled cell layers in carcinomas was 47 ± 8. Comparison of CD44 expression in samples of healthy mucosa, oral leukoplakia and carcinomas (Figure 2.23) showed a statistically significant difference between these three values ($p < 0.0001$). In contrast, the number of CD44-labelled epithelial layers at the margins of carcinomas did not differ from healthy mucosa.

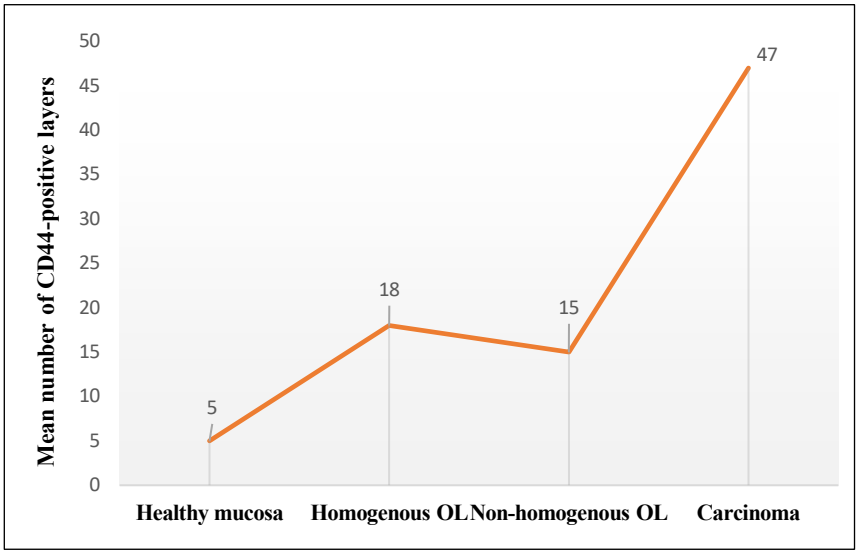


Figure 2.23 **Mean number of CD44-positive epithelial layers in healthy mucosa, oral leukoplakia and squamous cell carcinoma**

CD44 glycoprotein was also detected in mononuclear cells around the carcinoma, as well as in the intratumoral stroma. The reactivity around the tumour and the inflammatory infiltration of the intratumour did not correlate with the TNM and G values of the carcinoma ($p < 0.1$), probably due to the small number of analysed tumours. CD44 expression was also increased in inflammatory cells on the surface of carcinomas with ulceration at sites of mucosal necrosis (Figure 2.24 A and B).

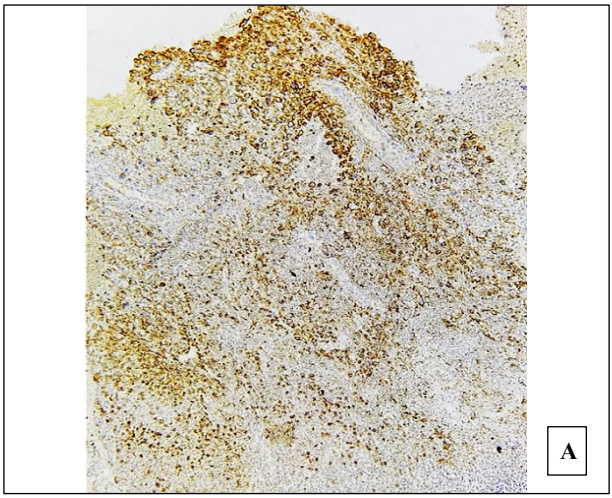
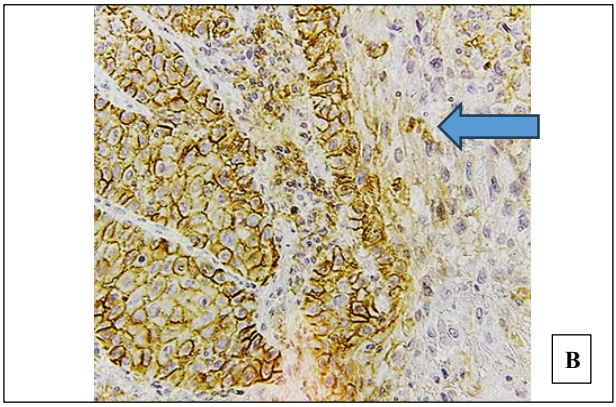


Figure 2.24 A **CD44 protein expression in detritus and inflammatory cells of ulcerating carcinoma**

Immunoperoxidase, anti-CD44, 100 × magnification.
Image by Madara Dzudzilo



2.24. B Figure. **Membranous expression of CD44 glycoprotein in moderately differentiated squamous cell carcinoma and rare stromal cells in the peritumoral zone** (arrow)

Immunoperoxidase, anti-CD44, 200× magnification.
Image by Madara Dzudzilo.

2.6 Immunohistochemical characterisation of CD9 antigen in oral mucosal tissues

2.6.1 Healthy oral mucosal tissue

CD9 antigen was expressed in the lower third of the morphologically intact oral mucosa, but not in the upper 2/3 of the mucosa. CD9 glycoprotein was detected in 3 - 8 mononuclear cells below the basement membrane. CD9 protein was also expressed in the salivary gland duct epithelium and in microparticles in their lumen (Figure 2.25).

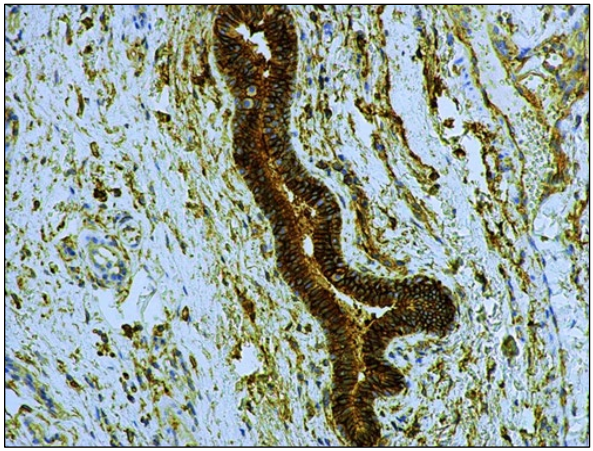


Figure 2.25 **CD9 antigen expression in the epithelium and microparticles in the lumen of salivary gland**

Immunoperoxidase, anti-CD9, 200 × magnification.
Image by Madara Dzudzilo

2.6.2 Homogeneous and non-homogeneous leukoplakias

In oral leukoplakia, CD9 expression in cell membranes was found in the same regions with the same number of epithelial cell layers as CD44 antigen expression. CD9 was not detected in the superficial layers of the oral leukoplakia

and in areas with keratohyalin granules. The pattern of CD44 expression in the cell membrane was as a straight line, whereas the presence of CD9 antigen showed slight thickening of the cytoplasm and membrane irregularities (Figure 2.26 A and B). The number of labelled epithelial layers in the centre of the homogeneous leukoplakia was 19, while 16 in the non-homogeneous one.

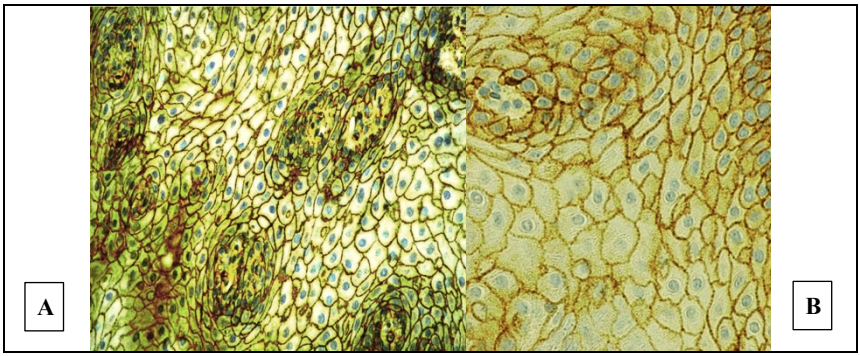


Figure 2.26 A un B **Immunohistochemical visualisation of CD9 antigen in epithelial cell membranes and exosomes**

Immunoperoxidase, anti-CD9, 200 × and 400 × magnifications.
Images by Madara Dzudzilo

In areas of mild and moderate dysplasia, CD9 protein expression is preserved in the epithelial cell membrane, although in severe and sometimes moderate dysplasia, we also found intracytoplasmic CD44 antigen expression (Figure 2.27).

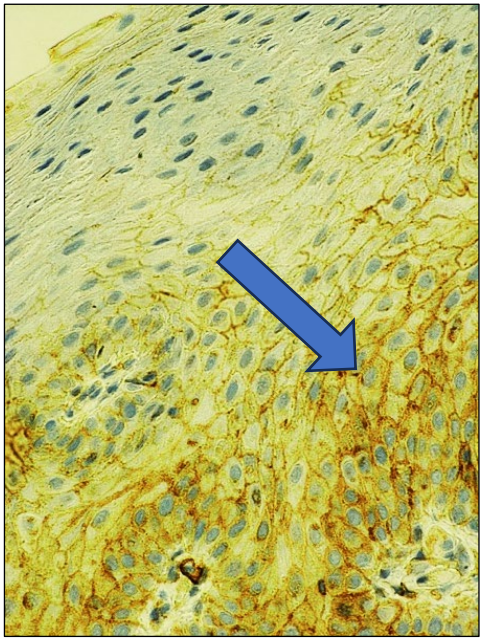


Figure 2.27 **CD9 antigen expression in epithelial cell membranes and cytoplasm in an area of moderate dysplasia** (arrow)

Immunoperoxidase, anti-CD9, 200 × magnification.
Image by Madara Dzudzilo

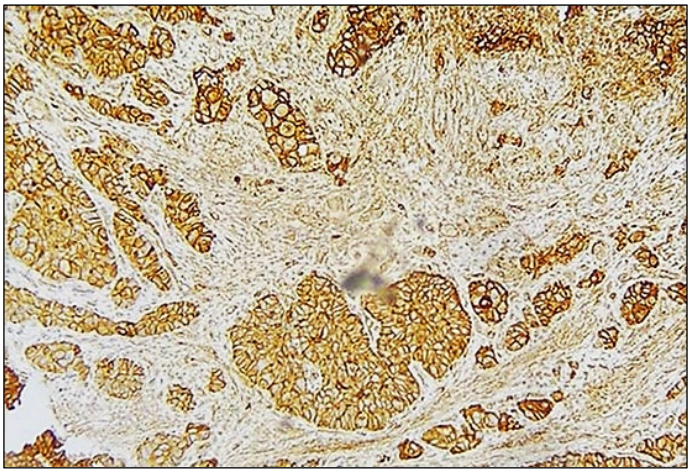
2.6.3 Oral squamous cell carcinomas

Of the 21 oral carcinomas, 20 have been evaluated, as one case was morphologically a salivary gland carcinoma with mucosal invasion. We diagnosed oral leukoplakia malignancy in two tissue samples.

Analysis of the comparative carcinoma group showed that the amount and type of CD9 glycoprotein expression was highly variable and we therefore divided all cases into three groups according to the type of CD9 expression:

1) preserved or partially preserved membranous expression (n = 5) (Figure 2.28);

2) CD9 is demonstrable in both cytolemma and cytoplasmic structures (n = 7) (Figure 2.29);
3) CD9 negative carcinomas (n = 8) (Figure 2.30).



2.28. Figure. **CD9 antigen expression in malignant cell membranes at the site of carcinoma invasion in the submucosa**

Immunoperoxidase, anti-CD9, 200 × magnification.
Image by Madara Dzudzilo

Due to the different expression patterns of CD9 glycoprotein, epithelial layers in the same field of view were not counted in tissue samples from this group. The total amount of CD9 antigen expression per carcinoma sample, compared to CD9 glycoprotein in healthy tissue and OL, is quantitatively increased at the expense of depth of malignant invasion and intracytoplasmic expression, except in the carcinoma group with complete absence of CD9 antigen.

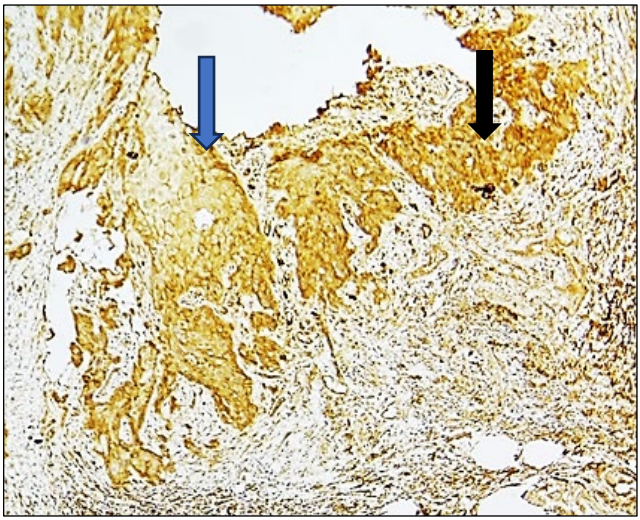


Figure 2.29 **CD9 antigen expression in carcinoma cell membranes** (blue arrow) **and cytoplasm** (black arrow)

Immunoperoxidase, anti-CD9, 200 × magnification.
Image by Madara Dzudzilo

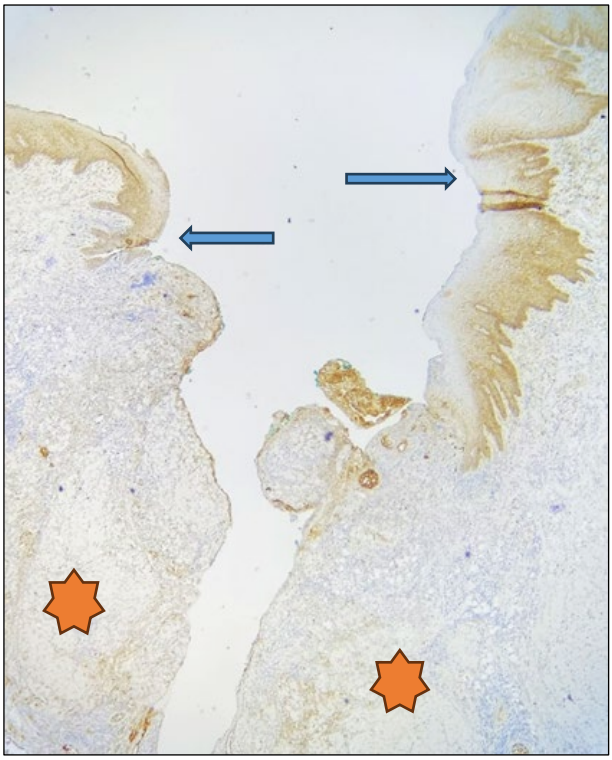


Figure 2.30 **Absolute lack of CD9 antigen expression in carcinoma along the salivary gland outlet** (asterisks)**, but the protein is well visualised on both sides of the cancer** (arrows)

Immunoperoxidase, anti-CD9, 100 × magnification.
Image by Madara Dzudzilo

The group analysed included areas of neoplasia with complete loss of CD9 antigen in the epithelium but preserved expression in plasma cell and lymphocyte aggregates around the carcinoma. In some carcinomas, CD9 expression was detected at the top of the tumour, but antigen levels decreased towards the invasion.

2.7 Immunohistochemical characterisation of ThPOK antigen in oral mucosal tissues

2.7.1 Healthy oral mucosal tissue

In healthy oral mucosal tissue, ThPOK antigen was expressed at all levels of the oral mucosa. The expression intensity ranged from weak to moderate and was observed in an average of 27 ± 5.6 labelled epithelial cell nuclei per field of view at 400-fold magnification, which we assessed in different mucosal layers (Figure 2.31).

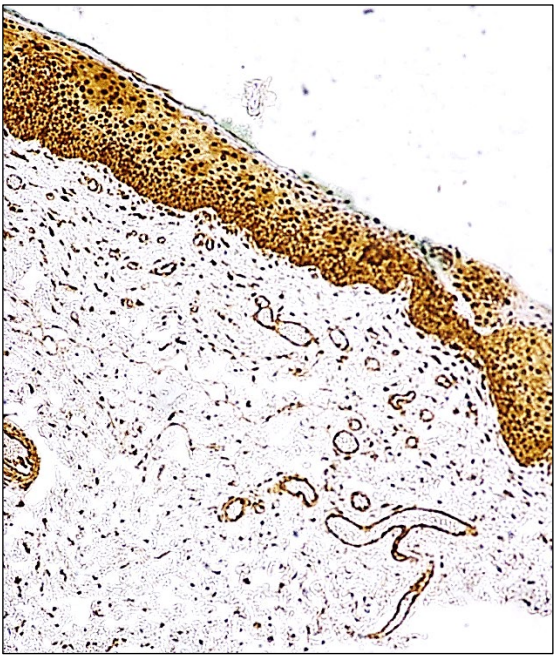


Figure 2.31 **ThPOK antigen expression in healthy oral mucosa**

Immunoperoxidase, anti-ThPOK, 100 × magnification.
Image by Madara Dzudzilo

2.7.2 Homogeneous and non-homogeneous leukoplakia

In oral leukoplakias, the ThPOK antigen was detected both in *str. basale*, both *str. spinosum*, *str. granulosum* and *str. lucidum* (Figure 2.32). The intensity of expression varied from weak to strong. ThPOK antigen positive immunoreaction was present in parakeratotic epithelial cells, but the in *stratum corneum* disappeared. In oral leukoplakia, the highest protein expression of ThPOK was in the dysplastic areas of verrucous leukoplakia, where the expression of the marker was also diagnosed in the squamous epithelium proliferates on its surface (Figure 2.33). The average number of epithelial nuclei with ThPOK labelling in leukoplakias was 43.7 % ± 9.4 %.

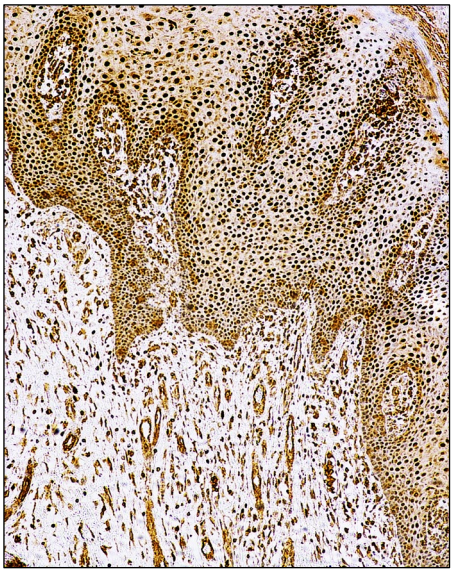


Figure 2.32 **ThPOK expression in the layers of oral leukoplakia tissue: *str. spinosum, str. granulosum* un *lucidum***

Immunoperoxidase, anti-ThPOK,
100× magnification
Image by Madara Dzudzilo

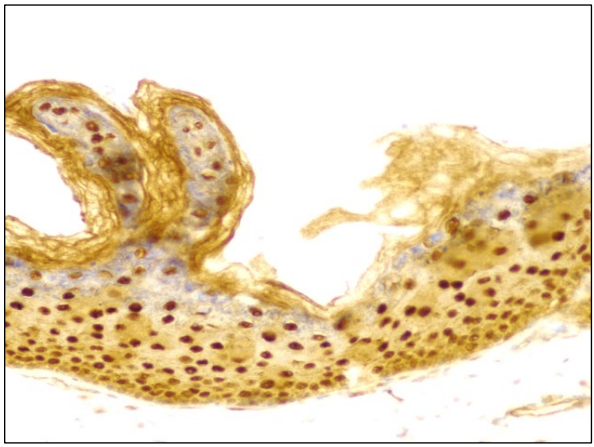


Figure 2.33 **ThPOK labelling throughout the OL thickness and in epithelial proliferates on the mucosal surface in verrucous leukoplakia**

Immunoperoxidase, anti-ThPOK 200 × magnification.
Image by Madara Dzudzilo

2.7.3 Oral squamous cell carcinomas

In oral mucosal squamous cell carcinomas, ThPOK was expressed throughout the depth of tumour invasion with a focal pattern (Figure 2.34 A and B). Its expression intensity varied from strong to weak, predominantly at level 2. The positive immune response disappeared in apoptotic cell groups and at sites of carcinoma overgrowth (Figure 2.35).

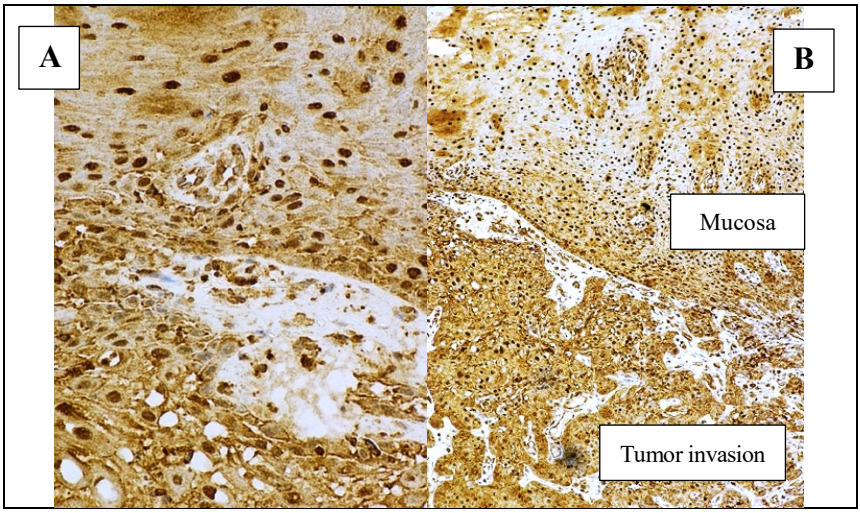


Figure 2.34 A un B **ThPOK moderate expression in the mucosa over and at the site of squamous cell carcinoma**

Immunoperoxidase, anti-ThPOK, 200 × and 100 × magnifications.
Images by Madara Dzudzilo

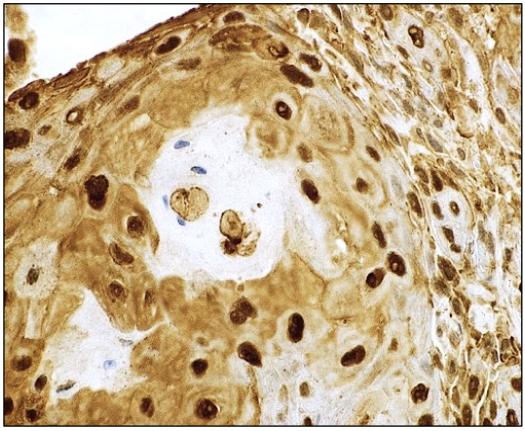


Figure 2.35 **Loss of ThPOK expression in keratinising area and apoptotic cells of oral squamous cell carcinoma**

Immunoperoxidase, anti-ThPOK 400 × magnification.
Image by Madara Dzudzilo

2.8 Immunohistochemical characterisation of p27 antigen in oral mucosal tissues

2.8.1 Healthy oral mucosal tissue

In healthy oral mucosa, p27 antigen was expressed only in the nuclei of epithelial cells. p27 protein was found in almost all cells *str. intermediale* and *str. superficiale*, but not found in *str. basale* (Figure 2.36). The median number of p27-positive cells in the comparator group was 76 (63 – 99) per field of view at 400 × magnification.

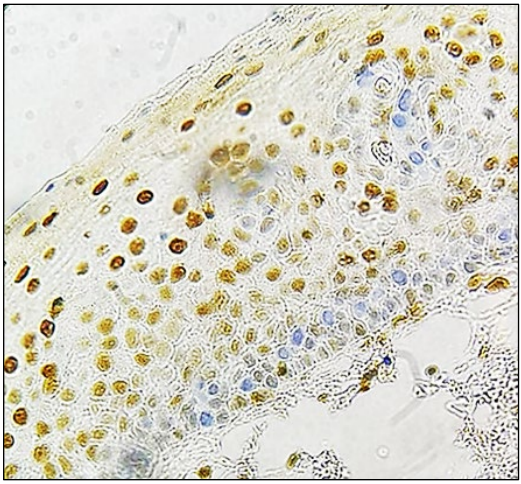


Figure 2.36 **Expression of p27 protein is absent in the basal layers of healthy oral mucosa**

Immunoperoxidase, anti-p 27, 200 × magnification.
Image by Madara Dzudzilo

2.8.2 Homogeneous and non-homogeneous leukoplakia

In both types of leukoplakia, the average number of p27-labelled cells per field of view at 400 × magnification was 111 (79 - 160). In the oral homogeneous leukoplakia, p27 antigen was expressed only in the nucleus of the epithelial cell

and was detected only in *str. intermediale* and *str. superficiale*. p27 protein expression was homogeneous and the intensity of nuclear staining was similar throughout (Figure 2.37).

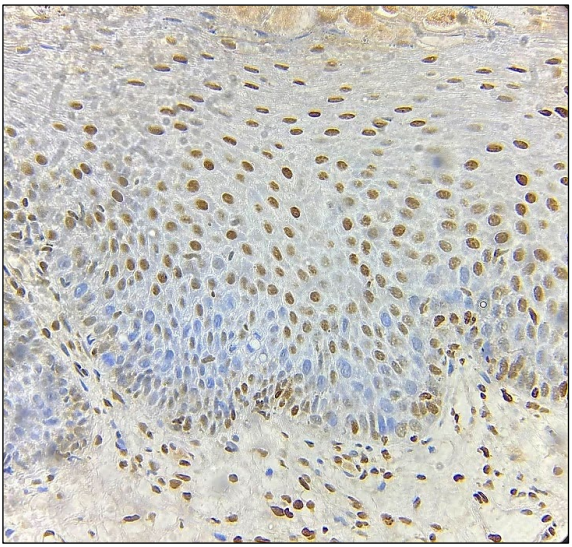


Figure 2.37 **Expression of p27 protein in nuclei of non-keratinised epithelium, predominantly in the upper layers of homogeneous leukoplakia**

Immunoperoxidase, anti-p27, 200 × magnification.
Image by Madara Dzudzilo

In non-homogeneous leukoplakia, p27 protein was expressed in *str. intermediale* and *str. superficiale* and in part of *str. basalis* and *str. parabasalis* (Figure 2.38). Loss of p27 expression was observed in epithelium with keratohyalin granules.

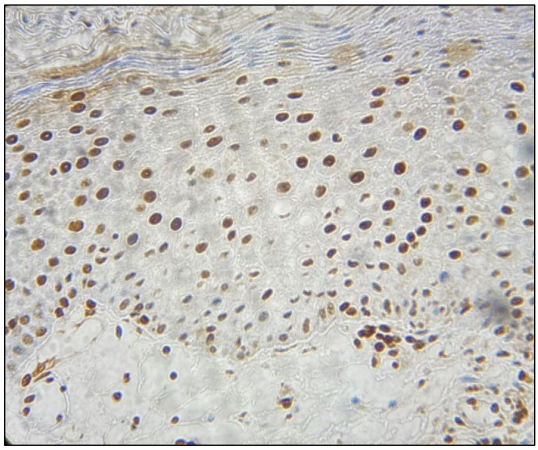


Figure 2.38 **Expression of p27 in all layers of non-homogeneous leukoplakia with moderate epithelial dysplasia**

Immunoperoxidase, anti-p27, 200× magnification.
Image by Madara Dzudzilo

The number of epithelial nuclei labelled with p27 antibody was lower in verrucous and nodular leukoplakia (mean 105 (75 – 163)) than in homogeneous leukoplakia (mean 120 (99 – 187)), but higher than in erythroleukoplakia (mean 75 (67 – 84)). A statistically significant difference ($p = 0.0002$) was demonstrated between p27 expression in healthy mucosa and in homogeneous oral leukoplakia. Considering the type of oral leukoplakia, a statistically significant difference ($p < 0.0001$) was found between homogenous and erythroleukoplakia (Figure 2.39). p27-positive nuclear expression decreased with increasing severity of the clinical form of leukoplakia: mean negative correlation $r_S = -0.352$ (Spearman rank correlation coefficient).

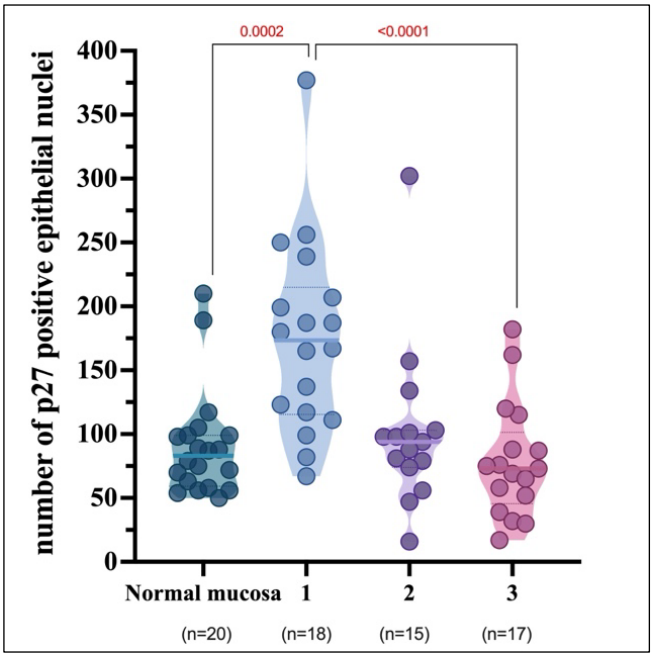


Figure 2.39 **Expression of p27 in epithelial nuclei of normal oral mucosa (comparison group) and various leukoplakia**

Leucoplakias: 1 – homogeneous, 2 – verrucous and nodular, 3 – erythroleukoplakia. Statistical significance (p, decimal places) determined by ANOVA followed by post-test (see Materials and Methods section)

Mosaic nuclear expression of p27 antigen was observed in erythroleukocytes. The p27 antigen is also expressed in some of the basal layer epithelial nuclei. However, in these OL, p27 protein was also frequently detected in the cytoplasm of the epithelium. (Figure 2.40). There was a moderate positive correlation ($r_S = 0.340$) between the number of p27-positive nuclei and the sex of the patient: the median number of p27-positive cells was 94 (75–114) in women and 127 (83–186) in men.

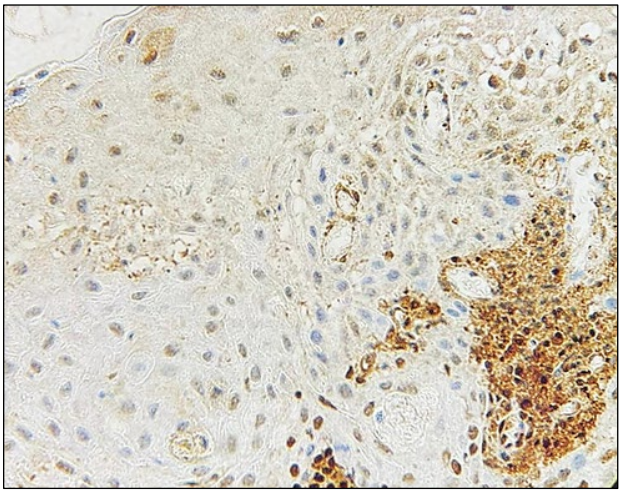


Figure 2.40 **Expression of p27 in nuclei and cytoplasm of non-homogeneous leukoplakia epithelium**

Immunoperoxidase, anti-p27, 200 × magnification.
Image by Madara Dzudzilo

2.8.3 Oral squamous cell carcinomas

The mean number of p27-positive cells in squamous cell carcinoma was 16 (10–36), which was significantly lower compared with the control group of 79 (63–99) ($p = 0.0002$) and all leukoplakia types of 111 (79–160) ($p < 0.0001$) (Figure 2.41).

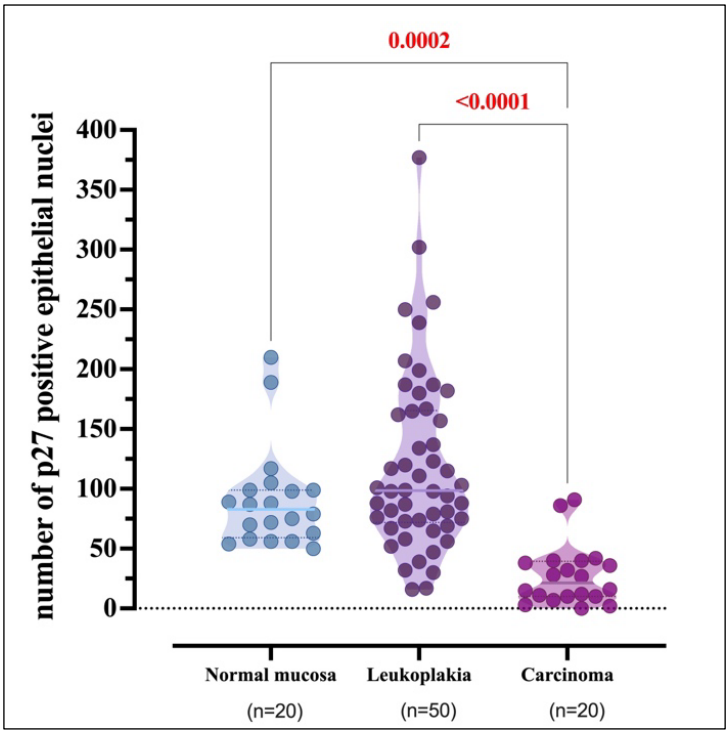


Figure 2.41 **Expression of p27 antigen in epithelial nuclei in healthy oral mucosa, leukoplakia and squamous cell carcinoma**

Statistical significance (*p*, decimal places) was determined by ANOVA followed by post-test (see Materials and Methods section)

In squamous cell carcinoma, expression of nuclear p27 was markedly reduced and focally disappeared, while expression of the antigen in the cytoplasm of carcinoma cells and in its stroma was confirmed simultaneously (Figure 2.42).

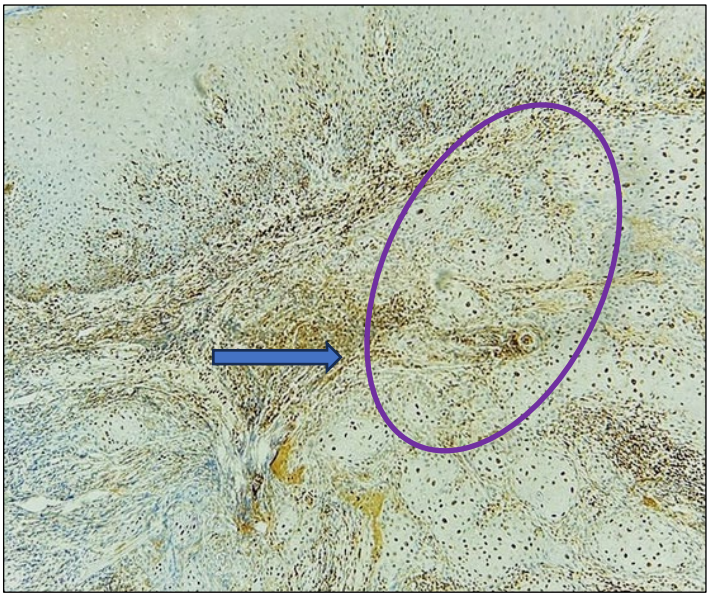


Figure 2.42 **Expression of p27 antigen in the nuclei of oral squamous cell carcinoma cells** (oval) **in the submucosa, but the protein is present in the cytoplasm of carcinoma cells and also in their stroma** (arrow)

Immunoperoxidase, anti-p27, 100× magnification.
Image by Madara Dzudzilo

In squamous cell carcinoma, p27 protein expression has a mosaic pattern and the intensity of antigen expression was visualised from light brown to dark brown.

Analysing the surgical material of a larger-sized oral mucosal squamous cell carcinoma, we found an increased expression of p27 protein in stem cells of the basal layer, but the expression gradually disappeared at the site of carcinoma invasion. We found a correlation of the amount of p 27 antigen expression with T-stages of carcinoma, compared to patients with normal oral mucosa (Figure 2.43 A). Correlation analysis with linear and non-linear tools revealed a strong negative correlation ($r_S = -0.9669$) for carcinoma stages (T).

This relationship was found with a non-linear quadratic polynomial model $R^2 = 0.9669$ (Figure 2.43 B).

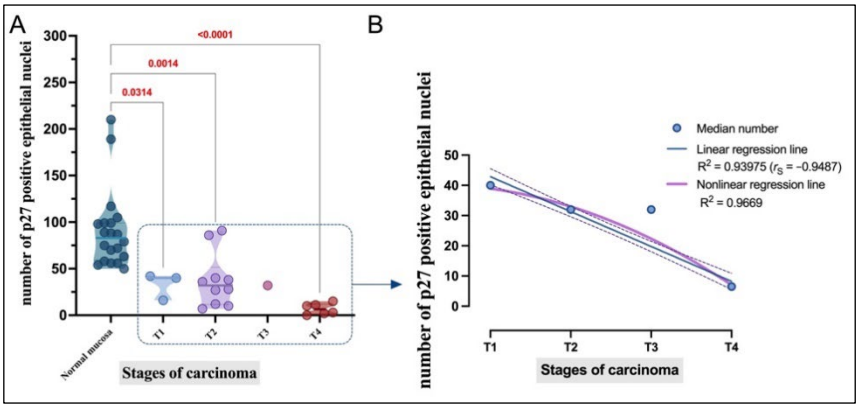


Figure 2.43 A. **Expression of p27 antigen in healthy oral mucosa and in tissue samples from patients with different stages of carcinoma;** B. **Correlation of p27 expression with carcinoma stages**

Statistical significance (*p*, decimal places) was determined by ANOVA followed by post-test (see Materials and Methods section); r_S – Spearman's rank correlation coefficient; R^2 – squared correlation coefficient

Discussion

Oral squamous cell carcinoma accounts for 2.1 % of all malignancies and is diagnosed in around half a million people worldwide each year (World Cancer Research Fund International). Oral carcinoma is diagnosed late, often developing metastases to cervical lymph nodes and distant organs, as well as recurrence. In Latvia, 214–218 people die from this oncological disease every year (WHO, 2020). It is therefore essential to diagnose and radically treat oral mucosal precancerous processes and to study the cellular and molecular mechanisms of their malignancy process.

The most common potentially malignant lesions of the oral mucosa are oral leukoplakia, oral erythroplakia, oral submucous fibrosis, oral *lichen planus* and actinic cheilitis (Mello et al., 2018). The global prevalence of potentially malignant oral lesions reported in the meta-analysis is 4.47 %. The most prevalent precancerous lesions in Western Europe are leukoplakia and oral *lichen planus* (4.11 %) (Mello et al., 2018).

In our study, homogeneous and non-homogeneous oral leukoplakias have been analysed clinically, morphologically and immunohistochemically in detail and their examinations have been compared with healthy mucosa and squamous cell carcinomas of the oral mucosa.

In European countries and the USA, oral leukoplakia develops into oral squamous cell carcinoma less frequently than in Asia (Alsalem, 2019; Narayan&Shilpashree, 2016; Speight et al., 2018). It is important to emphasise that potential precancerous oral lesions as well as malignancies of this localisation belong to self-investigable neoplasias, and patients should be involved in the detection of visual or palpable changes of the oral mucosa. The diagnostic role of the various subspecialties is crucial, as they must differentiate leukoplakia from other 'white' and 'red' epithelial and non-epithelial mucosal lesions of the oral cavity. Therefore, potentially malignant lesions of

the oral cavity are confirmed only by microscopic or cytological examinations of biopsies and surgical specimens.

The age of the patients included in our study ranged from 27 to 82 years, with a mean age of 57 years. The average age range of 56–58 years has also been reported by other authors in their studies (Evren et al., 2023; Gandara-Vila et al., 2018; Georgaki et al., 2021; Rubert et al., 2021; Venkat Naga et al., 2019).

The study was predominantly male (1.3:1 female ratio), which is consistent with the findings of several other authors on the higher prevalence of OL among men (Dogenski et al., 2021; Ghazi et al., 2021; Neville et al., 2002; Stojanov et al., 2024). However, it should be noted that at the same time, a large proportion of studies in Western countries point to an increasing trend in the incidence of OL among women (Evren et al., 2023; Georgaki et al., 2021; Napier et al., 2003; Rubert et al., 2021; Shavlokhova et al., 2021).

We compared the clinical results obtained with literature data based on the classification of leukoplakia used in Western patient databases, as oral leukoplakia may have slightly different characteristics and prognosis compared to Asian countries due to differences in diet, tobacco use and possibly genetic differences (van der Waal, 2018).

In our study patients, clinical OL was predominantly located in the buccal mucosa (n = 18), followed by the lateral and ventral surface of the tongue (n = 17). Other affected sites were the floor of the mouth, gingiva and lip mucosa. However, according to the literature, the tongue (ventral and lateral surfaces) was the most commonly affected localization (55.4 %), followed by the buccal mucosa (13.8 %), the floor of the mouth (8.8 %), gingiva (8.7 %), lip (4.1 %) and palate (2.2%) (Aguirre-Urizar et al., 2021; Nagao et al., 2016; Neville&Day, 2002). In contrast, Warnakulasuriya and Ariyawardana (2018) found in their systematic review that globally, the buccal mucosa was the most common site for OL overall (18.4 %), yet it had the lowest rate of malignant transformation

(3.35 %) (Warnakulasuriya et al., 2016). On the other hand, 16.14 % of OLs were localised in the tongue, while the malignant transformation (MT) rate reached 24.22 %. Some authors have also indicated the predominant localisation of OL in the buccal mucosa in their studies (Bagan et al., 2022; Dogenski et al., 2021; Okut et al., 2019; Tovaru et al., 2023). Other authors (Abati et al., 2020; Tamatani et al., 2018) in the literature indicate the presence of multiple leukoplakia in a single patient. In our study, we observed the presence of multiple leukoplakia in only 6 patients.

Clinically, leukoplakia has been divided into homogeneous and non-homogeneous leukoplakia for decades, with the latter characterised by different subtypes (van der Waal, 2018). Among our diagnosed leukoplakia, the following clinical variants of leukoplakia were present: homogeneous leukoplakia n = 18 (36 %) and non-homogeneous leukoplakia n = 32 (64 %). Among the non-homogeneous leukoplakia, erythroleukoplakia (n = 17), verrucous leukoplakia (n = 11) and nodular leukoplakia (n = 4) were identified. In the literature, in studies conducted in European countries, the distribution of homogeneous OL ranged from 42 % to 82 % and of non-homogeneous OL – from 18 to 58 % (Evren et al., 2023; Gandara-Vila et al., 2018; Jäwert et al., 2021; Rubert et al., 2021). However, for example, Okut et al. (2019) indicate a dominance of the non-homogeneous form (64 %) compared to the homogeneous one. The authors also point out in their studies the diagnosis of different subtypes of non-homogeneous leukoplakia. For example, in their study, Okut et al. (2019) found predominantly a nodular form 18 (22.8 %), then verrucous 9 (11.4 %), while Rubert et al. (2021) and Tovaru et al. (2023) found a predominant verrucous form, 39 (9.5 %) and 35 (29.2 %) respectively, then erythropleukoplakia n = 12 (10 %) and nodular 1 (10.8 %) (Tovaru et al., 2023).

The size of leucoplakia can vary greatly. In our study, they varied in diameter from 4 mm to 30 mm, with a mean size of 14 mm ± 3.7 mm.

Leukoplakia were smaller in females – 5 mm, while in males the mean largest OL diameter was 27 mm.

Oral leukoplakia larger than 2 cm were present in 44 % (n = 22) of patients, but it should be emphasised that they were operated and the risk of malignancy was avoided, as according to studies by other authors OL larger than 2 cm are highly potentially malignant growths in the oral mucosa (Assimakopoulos et al., 2002; Holmstrup et al., 2006; Speight et al., 2018). Rubert (2021) found in his study that the mean lesion diameter was 18.64 ± 14.92 mm, with non-homogeneous leukoplakia lesions being significantly larger (mean 22.63 mm). In a study by Brouns et al. (2014) concluded in their 2014 study that large lesion size (≥ 4 cm) was the only statistically significant predictor of malignant transformation; however, their 2023 study concluded that there was no statistically significant association between lesion size and MT (Brouns et al., 2014). In our opinion, it is important to consider not only the diameter of the potentially malignant mass, but also its thickness. Thicker oral leukoplakia have impaired epithelial maturation and differentiation, poorer blood supply to multiple epithelial layers, altered cell cycle and more frequent organelle damage, thus more likely to develop and progress to dysplasia (Brizuela et al., 2023; Dzudzilo et al., 2021).

The visually invisible but altered borders of potentially malignant growths and tumours in the mouth vary in size and are irregular. The surgical site is defined within 10 mm of the outer border of the mass (if the OL localisation allows), but this does not ensure 100 % removal of the lesion. Therefore, new examination and visualisation techniques are being developed to allow the surgeon to more accurately determine the primary lesion boundaries before and/or during surgery (Pošta et al., 2023). One of the advanced examination methods was also applied in our study. Direct fluorescence with the VELscope device is a screening method for better assessment of potentially malignant

masses. VELscope illumination induces the excitation of endogenous fluorophores in the mucosa and submucosa, which emit green light. The visible loss of physiological fluorescence indicates epithelial dysplasia but can also be observed in hyperaemia, traumatisation, hyperkeratosis and other benign changes, which reduces the specificity of the method (Elvers et al., 2015; Koch et al., 2011; Pošta et al., 2023). We also found a loss of fluorescence in the mucosa of our control subjects in areas with local inflammation. A drawback of this method is the ability to detect changes only in the superficial mucosal layers. Elvers et al. in their study, like us, noted the importance of the fluorescence method in the delineation of oral leukoplakia. They demonstrated that oral leukoplakia is 66 % smaller visually than it is visualised through a VELscope device (Elvers et al., 2015). The real boundaries of the lesions are wider than those visually visible. In contrast, Amirchaghmaghi et al. in a study of oral squamous cell carcinomas evaluated with the VELscope device found no significant differences in colour intensity between tumours with different depths of invasion (Amirchaghmaghi et al., 2023). The method has a sensitivity of 70.19 % and a specificity of 65.95 % (Cicciù et al., 2019), so the lack of specific criteria limits the interpretation of VELscope results.

Warnakulasuriya et al. (2016) found that the determinants contributing to the malignant potential of OL are increasing age, female sex, leukoplakia exceeding 200 mm^2 and belonging to a non-homogeneous type (e.g. erythroleukoplakia) with increasing degrees of dysplasia (Warnakulasuriya&Ariyawardana, 2016). Aguirre-Urizar et al. (2021) suggested that female sex, clinical type of erythroleukoplakia and presence of epithelial dysplasia were significantly associated with MT (Aguirre-Urizar et al., 2021). As our study took place between 2016 and 2022, we considered the 2017 WHO recommended classification of dysplasias for the assessment of dysplasias. Therefore, we distinguished mild, moderate and severe dysplasia. The new WHO

Classification of Tumours of the Head and Neck, 5th Edition, Volume 9: *Head and Neck Tumours* (WHO, 2024) recommends to distinguish only mild and severe dysplasia. The need to target a detailed description of cellular changes in the dysplasia score and to distinguish between the 3 grades of dysplasia is also supported by the current COST project "Interceptor oral cancer", No CA21140 (2022–2026), which aims to study early molecular changes in leukoplakia specifically with features of mild and moderate dysplasia.

In addition to clinical and several histopathological factors, the risk of malignant transformation is particularly dependent on the form and degree of dysplasia (Speight et al., 2018). Gandara-Vila et al. (2018) concluded that the presence of dysplasia is the only risk factor statistically associated with the development of carcinoma (Gandara-Vila et al., 2018). In our study group, dysplasia was present in 66 % of cases, of which 12 (24 %) had mild dysplasia, 8 (16 %) moderate and 13 (26 %) severe dysplasia. Studies indicate that the risk of MT is higher in moderate and severe dysplasia compared to mild dysplasia and OL without dysplasia (Iocca et al., 2020; Warnakulasuriya&Ariyawardana, 2016). Warnakulasuriya et al. (2011) reported in their study the rates of OL in mild dysplasia, moderate dysplasia and severe dysplasia as 4.8 %, 15.7 % and 26.7 %, respectively, confirming that the severity of dysplasia plays a role in the prognosis of carcinoma (Warnakulasuriya et al., 2011). In their studies, several European authors report the presence of oral epithelial dysplasia in 8–40 % of homogeneous leukoplakia and 25–77% of non-homogeneous OL (Dost et al., 2013; Rubert et al., 2021; Speight et al., 2018). Severe dysplasia was significantly more common in patients with non-homogeneous lesions (Rubert et al., 2021). We can confirm the same, as we found the presence of severe dysplasia only in erythroplakia in n = 13 (26 %).

In our study, we found hyperplasia in n = 11 (34%) and mild dysplasia in n = 7 (24 %) homogeneous leukoplakia. Rubert et al (2021) made a similar

conclusion in their study that histologically, most lesions (n = 271; 65.7 %) did not show dysplasia, but among dysplasias (n = 141; 34.3 %), mild dysplasia was the most common (n = 98; 23.8 %). The majority of homogeneous OL lesions had no epithelial dysplasia (92.5 %). Okut et al. (2019), on the other hand, histologically observed predominantly squamous epithelial hyperplasia, which was present in n = 17 (34 %) patients in our case.

However, it should be noted that although there are a number of predisposing factors for carcinoma development, this does not mean that homogeneous leukoplakia or leukoplakia without dysplasia cannot develop into cancer, as specifically pointed out by (Villa et al., 2017). This is demonstrated by the study of Bagan et al. in 2022, which concluded that 7.2 % of homogeneous leukoplakia developed into carcinoma and 7.7 % of leukoplakia without dysplasia transformed into carcinoma, while MT occurred in 25.9 % with mild dysplasia, 36.4 % with moderate dysplasia and 50 % with its severe form.

In Western countries, smoking and alcoholism are the main risk factors for the development of oral precancers and, eventually, carcinomas. This is in contrast to South Asia and the Pacific, where betel nut chewing and leaf smoking are major factors in the development of leukoplakia and erythroplakia and oral carcinomas. According to a study by Dogenski et al. (2021), the most likely etiological factors were smoking (44.7 %) and alcohol consumption (9.85 %) (Dogenski et al., 2021). Oral leukoplakia caused by alcohol and/or tobacco use is usually asymptomatic, given an evolutionary time of less than 12 months. It is well known that smoking also affects the oral leukoplakia microenvironment. Chemicals in tobacco smoke cause chronic inflammation in the oral mucosa, which contributes to an immunosuppressive microenvironment. A team of scientists led by Yagyuu (2021) showed that non-smoking patients are less likely to develop oral leukoplakia than smoking individuals (Yagyuu et al., 2021). However, if oral leukoplakia develops in non-smokers, the risk of malignant

transformation is higher. Tobacco smoking is one of the most important risk factors for the development of oral carcinoma, whether or not a precancerous lesion such as oral leukoplakia develops.

In addition to traditional risk factors for oral carcinomas, human papillomaviruses (HPV) also have important effects (Barsouk et al., 2023). In the context of the new WHO classification of head and neck tumours, fifth edition, oral squamous cell carcinomas are divided into HPV-associated and non-HPV-associated. It should be noted that HPV involvement is observed in 6 % of oral carcinomas (Katirachi et al., 2023) and up to 70 % in oropharyngeal carcinomas (Timbang et al., 2019), thus a greater role for HPV in the development of oropharyngeal carcinomas. According to the new classification, the association of HPV with oral dysplasias is similarly assessed. As our study took place between 2016 and 2022, we followed the classification of dysplasias and carcinomas recommended in the Classification of Head and Neck Tumours, 4thedition (El-Naggar et al., 2017).

Despite studies by dentists, oral medicine specialists, geneticists, immunologists and pathologists, the malignant process of oral leukoplakia is undoubtedly long, multi-step, rather controversial and not fully understood. Several immunohistochemical markers have been historically used to study oral leukoplakia: p53, p16, Ki67, cytokeratins, vimentin and others (Cema et al., 1998; Choi et al., 2003; Kresty et al., 2008; Sridharan et al., 2016; Visioli et al., 2012).

This means that it is important to identify other markers that could indicate the presence of early molecular changes in cancerous tissues such as oral leukoplakia. We therefore choose to study cell adhesion, stem cell, cell cycle and transcriptional regulator proteins with biomarkers CD44, CD9, ThPOK and p27.

The CD44 protein has been analysed in various oral mucosal pathologies for more than three decades, but recent studies on the CD44 antigen in the oral mucosa have become more relevant as it can also be detected in saliva. Our

results showed that the colour of the test device changed from yellow to dark green with increasing levels of total protein, which was indicated by the numbers 1, 2, 3 and 4 respectively. The figures indicate that the OncAlert® Oral Cancer Rapid test provides an indication of a low or increased risk of malignant transformation in leukoplakia. A statistically significant difference was found between higher total salivary protein levels and more clinically severe forms of oral leukoplakia (non-homogeneous form, erythroleukoplakia type).

Our study showed an increase in the intensity of the SolCD44 test when CD44 antigen was expressed in leukoplakia tissue not only in the oral epithelial cell membrane, but also in its cytoplasm and in mononuclear cells under the OL. Similar data were obtained by the laboratory led by E.R. Cohen (Cohen et al., 2020) by immunohistochemical evaluation of CD44 expression in oral and oropharyngeal carcinomas, as well as in SolCD44 oral rales, but this was measured by ELISA test method. The authors found a significant correlation between the level of SolCD44 in saliva and CD44 expression in tissues. Significant correlations between CD44 expression in biopsy specimens and levels of soluble CD44 in saliva have also been found in oral *lichen planus* patients (Chaiyarit et al., 2008).

When assessing total protein, it should be borne in mind that saliva is a complex fluid containing a wide range of biomolecules such as proteins/peptides, nucleic acids, electrolytes, hormones from both local and systemic sources, enzymes, antibodies, antimicrobial components and cytokines. Most of the organic compounds in saliva are produced locally in the salivary glands, but some molecules from the blood enter the saliva. Biomolecules can enter saliva by diffusion, filtration and/or active transport (Wang et al., 2019). As mentioned above, saliva contains not only secretions from large and small salivary glands, but also sloughed epithelial cells from normal mucosa and leukoplakia tissue. Other investigators have highlighted the importance of

SolCD44 and total protein levels as a useful diagnostic marker for oral, head and neck squamous cell cancers prior to verification of the pathohistological pattern (Metgud et al., 2014; Pereira et al., 2016).

Currently, there are only selected groups of researchers using the OncAlert® Oral Cancer Rapid test. However, studies have drawn attention to the fact that an increase in SolCD44 in saliva indicates an initial dysplasia in the tissue. OncAlert® test users note, however, that the inflammatory process in the mucosal tissue can cause false-positive results (Franzmann et al., 2018).

CD44-pan antigen is one of the adhesion molecules that implement cell-to-cell interactions in the oral mucosa, including in leukoplakia, but it has also been shown to be altered in processes such as inflammation, neoplasia and autoimmune diseases (Puré et al., 2001). The CD44 protein has a molecular mass of 85 – 90 kDa and is composed of 1 – 5 and 16 – 20 exons transcribed. Oral researchers mainly emphasise the role of CD44 as a prognostic factor for oral squamous cell carcinoma. Some research groups have found that overexpression of CD44 correlates with more extensive metastasis, higher T stage and lower differentiation G stage (Negi et al., 2012; Ortiz et al., 2018), while other researchers have highlighted that reduced CD44 levels are not associated with oral carcinoma progression (Boxberg et al., 2018). Most authors describe the localisation of the CD44 adhesion molecule in cell membranes, and only a few have diagnosed its expression in the cytoplasm (Groma et al., 2012; Kaza et al., 2018). In oral leukoplakia, results on CD44 antigen are even more controversial, as studies have used different microscopic magnifications during pathohistological examinations, described results in fields of view of different sizes and used highly variable methods to assess antigen expression. In our study, we expressed the amount of CD44 by the number of labelled epithelial layers in healthy oral mucosa, OL and carcinoma, as this more objectively reflects the amount of adhesion molecules in leukoplakia and oral carcinomas of different

thicknesses. Statistical analysis of our results showed a statistically significant difference between the number of CD44-labelled layers in normal mucosa and oral leukoplakia tissue. Therefore, we recommend this method of CD44 protein evaluation to better assess CD44 expression in studies of potential precancerous lesions and early oral carcinomas. Our study demonstrated CD44 intracytoplasmic immunoexpression in 47 % of non-homogeneous oral leukoplakia cases as well as in squamous cell carcinomas. Such atypical CD44 localisation has been noted predominantly in studies of oral squamous cell carcinoma (Khajuria et al., 2015). Authors have mentioned this expression site due to the interaction of CD44 antigen with the cell cytoskeleton (Harada et al., 2007; Miletti-González et al., 2012). In our opinion, this fact probably reflects the expression of the glycoprotein CD44 in damaged mitochondria, ribosomes, endoplasmic reticulum and/or Golgi complex of the hyperplastic and dysplastic epithelium of oral leukoplakia, but this needs to be clarified by electron microscopic examinations of leukoplakia. In the last decade, the CD44 antigen has been named as a marker of stem cells in various organs (Jakovlevs et al., 2019; Pettersen et al., 2011; Tamatani et al., 2018). Therefore, it is clear that we demonstrated CD44 glycoprotein in the membranes of carcinoma, actively proliferating and mature oral mucosa cells. However, it is important to stress that there are three types of stem cells: embryonic, mature and cancer cells. In oral leukoplakia and carcinomas, there are likely to be variants of mature and cancer stem cells, now also referred to as tumour-initiating cells (Venkat Naga et al., 2019).

The intracytoplasmic expression pattern of CD44 in oral epithelium can be used as a prognostic factor for the eventual transformation of non-homogeneous leukoplakia into early intraepithelial carcinoma. The distinction between mature stem cells and so-called cancer stem cells is so fragile, unstable and difficult to prove by immunohistochemical studies alone that the study of oral leukoplakia must be complemented by its genetic

investigation (Ambele et al., 2020). The CD44 antigen can bind growth factors, hyaluronic acid, collagen, fibronectin and metalloproteinases, and CD44 has been described to be involved in processes such as lymphocyte and macrophage activation and leukocyte aggregation (Alves et al., 2009; Gore et al., 2008). Importantly, our results showed a statistically proven difference between the amount of CD44 labelled mononuclear cells in normal mucosal and leukoplakia tissues. CD44 glycoprotein was also detected in mononuclear cells around the carcinoma, including intratumoral stroma, but reactivity around the tumour and intratumorous inflammatory infiltration did not correlate with carcinoma TNM and G values due to the small number of tumours. An increased number of mononuclear cells (macrophages and lymphocytes) under the basement membrane was found in oral leukoplakia compared with normal oral mucosa. Our statistical analysis showed a moderate correlation between the number of CD44-positive mononuclear cells under the basement membrane and the number of CD44-labelled oral epithelial layers in the central part of the leukoplakia, where the interaction between cell adhesion molecules is likely to be most active in both the epithelium and the mesenchyme. The simultaneous accumulation of CD44 antigen in epithelial and stromal cells of leukoplakia and carcinoma has led to its involvement in local immune responses, long-term epithelial-mesenchymal interactions as hyaluronic receptor and antiphagocytic proteins.

In addition to CD44 antigen analysis, tetraspanin CD9 was also evaluated as a marker of exosomes in the cytolemma. Although there is increasing interest in extracellular vesicles that have been studied in oral leukoplakia, few studies on CD9 expression in oral precancerous lesions and epithelial dysplasias are available in the literature (Li et al., 2019; Nankivell et al., 2013).

To test our hypothesis of its involvement in two important oral mucosal processes, CD9 antigen analysis was performed. The first is that this surface

protein participates in the interactions between multiple cells as well as between cells and the extracellular matrix, allowing the transport of SolCD44 and other fluids to the oral mucosal surface. Therefore, the finding that both CD44 and CD9 antigens are expressed in the membranes of oral mucosal epithelial cells in healthy mucosa, oral leukoplakia and oral squamous cell carcinoma is of scientific and clinical interest. The expression pattern of CD44 and CD9 differed: CD44 protein in oral mucosal epithelium is visualised as a thin, smooth line in the cell membrane, whereas CD9 is expressed as a jagged line in the membrane with thickening, which may correspond to exosomes/microvesicles (Peng et al., 2020; L. Wang et al., 2019; Xiao et al., 2018). CD9-positive microenvironments of the oral epithelium are likely to be so-called transport or channel proteins and explain the fluid transport process in healthy mucosa, leukoplakia and carcinoma. CD9 is also known to mediate intercellular communication between cells (Baghban et al., 2023; Xing et al., 2020) and we believe that this also occurs between malignant cells, except in our patient group with total CD9 loss in 40 % of carcinoma cases.

The second aim of the parallel study of CD44 and CD9 proteins was to investigate whether CD9 expression is altered during malignancy of oral leukoplakia. Some researchers suggest that a decrease in CD9 expression correlates with malignant transformation of oral leukoplakia (Nankivell et al., 2013), while a 2019 study by Wang (L. Wang et al., 2019) concluded that CD9 inhibits the progression of potentially malignant processes. Our results showed a trend towards a decreased number of CD9-labelled epithelial layers in non-homogeneous leukoplakia compared with homogeneous ones. In a study by Baghban et al. (2023) report the opposite theory that exosomes can promote cancer cell proliferation: cancer cells can release exosomes containing molecules. These can help prepare the tumour microenvironment to be more susceptible to metastasis. For example, exosomes can transport enzymes that

help degrade the extracellular matrix surrounding the primary tumour, facilitating the invasion and spread of cancer cells to other parts of the body (Baghban et al., 2023; Yousafzai et al., 2021).

In oral leukoplakia, the malignant process is undoubtedly slow, lasting up to 7 years according to scientific studies (Aguirre-Urizar et al., 2021; Mello et al., 2018), making it particularly important to assess nuclear changes by immunohistochemical methods in addition to cytochemical markers. Among many antibodies, we chose to analyse the less known ThPOK and the already validated p27 protein to study the malignisation process of oral leukoplakia.

One of the most controversial immunohistochemical markers is ThPOK. The protein ThPOK 67 (ZFP67) with zinc epitopes consists of 539 amino acids and has a molecular weight of 58/80 kDa. The presence of ThPOK is essential for other oncogenes to initiate the carcinoma process (Maeda et al., 2005). This protein regulates transcription and is involved in biological processes such as DNA damage, embryonic development, cell cycle control, cell proliferation and differentiation. Initial studies on ThPOK were in large B-cell, follicular and non-Hodgkin lymphomas (Jiang et al., 2019; Liu et al., 2017). Subsequent publications were on the study of ThPOK expression in lung, liver, breast, ovarian and prostate carcinomas (Maeda et al., 2005; Zu et al., 2011). For example, in breast carcinoma, it is likely that a protein with zinc epitopes regulates the expression of several genes involved in signal transduction and metabolic processes, promoting tumour growth and progression by inducing the expression of the apoptosis-inhibiting protein surviving (Zu et al., 2011). A group of scientists, Jiao et al. (2013) conducted a study of ThPOK expression in nasopharyngeal carcinoma cell cultures and tissues, where it was found that protein expression was significantly higher in undifferentiated non-keratinized nasopharyngeal carcinoma tissue compared to mucosa affected by chronic rhinitis (Jiao et al., 2013). To date, only one study on ThPOK expression in oral

carcinoma is known (Sartini et al., 2015), in which the authors compared the expression of the marker in normal mucosa and squamous cell carcinoma tissue and found a decrease of the antigen in the malignancy. The published data are in contrast to the results of ThPOK expression in carcinomas of other localisation: breast tumours (Zhang et al., 2018; Zu et al., 2011), nasopharyngeal carcinomas (Jiao et al., 2013), where most researchers noted an increase in expression of ThPOK protein with zinc epitopes.

In our cohort of subjects, ThPOK antigen has been shown to be present in all mucosal layers in healthy oral mucosa and OL: both basal, where the cell division zone is active, and suprabasal, where more mature epithelium is localised. In oral leukoplakia, ThPOK was expressed both in hyperplasia and dysplasia. The PubMed literature database available to us does not contain any publications on ThPOK expression in OL. The dynamics of the number of labelled nuclei is increasing from unaltered mucosa to OL and to the comparative carcinoma group: 27 ± 5.6 : 43 ± 9.4, respectively: 49 ± 7.8 with a statistically significant difference between normal and OL and between normal and carcinoma, but no statistical significance between ThPOK OL and carcinoma, which could be explained by the loss of transcriptional properties in atypical undifferentiated squamous epithelial cells, visualised by the disappearance of pericellular antigen in immunohistochemical preparations. However, we cannot agree with the findings of Sartini et al. (2015) that 36 % of cancers were totally negative for ThPOK expression, suggesting that there is no transcription between DNA and matrix RNA in malignant epithelial cells. Studies on Th POK in the last five years have tended to show that this protein acts more frequently on immune cells in malignant tumours and thus affects the prognosis of neoplasia (Xia et al., 2021; Yang et al., 2022).

However, it should be emphasised that the oral mucosal epithelium is ectodermal, whereas the others are of entodermal origin, so it would not be

academically valid to compare the results of ThPOK studies in our analysis group with breast, prostate, gastric carcinomas or lymphomas, where some of the results were obtained by flow cytometry or Western blot. Numerous in vitro and in vivo studies confirm that ThPOK controls key "fate" decisions of erythroid, lymphoid, osteoclast, adipocyte, epithelial and CNS cells (Lunardi et al., 2013) by acting as both an oncogene and an oncosuppressive agent (Constantinou et al., 2019; Zu et al., 2011). It is the dual nature of this protein with zinc epitopes that prevents us from recommending this antigen as a prognostic marker in oral leukoplakia malignisation.

The other nuclear marker we analysed in our study was the p27 protein. The results of our study showed that p27 antigen is present in a fairly broad spectrum of epithelia: healthy oral mucosa, hyperplasia, dysplasia and oral squamous cell carcinoma tissues. In this study, we have analysed p27 protein in homogeneous, non-homogeneous oral leukoplakia and compared its abundance in carcinoma. There are few studies on p27 protein expression in different clinical types of oral leukoplakia with accurate estimation of its nuclear immunopositivity, as well as on p27 antigen expression in the epithelial cytoplasm in OL and cancer (Kövesi et al., 2006; Queiroz et al., 2010; Vallonthaiel et al., 2016). As the main potentially malignant oral neoplasms, non-homogeneous leukoplakia cases were of particular clinical relevance for detailed statistical analysis of p27 protein abundance.

The highest amount of p27 protein is present in the 'G_1' stage of the cell cycle to realise entry into the 'S' phase, where DNA replication and intense protein synthesis occur. We can state that in the epithelium of homogeneous leucoplakia the cell cycle is intact, as dysplasia is rarely observed. So, as long as the p27 protein is present in the nucleus, we know that the oral epithelium will grow and divide in a controlled manner and that it will circulate in a time frame that is specific to each site of the oral mucosa. There is a statistically significant

difference between the increase in the number of nuclei of labelled cells in healthy mucosa and in homogeneous leukoplakia. We believe that this is due to the fact that in homogeneous leukoplakia, hyperplastic processes predominate and the p27 protein still acts as a tumour suppressor. However, we cannot agree with the authors who conclude that p27 is not expressed in all leukoplakia but only in a proportion of leukoplakia cases and show the results as a percentage. For example, different groups of investigators report that p27 antigen expression ranges from 40 % to 78 % (Kudo et al., 1998; Queiroz et al., 2010; Tsuzuki et al., 2003). We confirm that the p27 protein is present in all tissue samples of intact mucosa, leukoplakia and oral carcinomas. The only questions are how much of this protein is present, how stable is it and where is it located in the cell?

Our results show that the number of p27 antigen-expressing cells tends to decrease from homogeneous to non-homogeneous leukoplakia. However, a statistically significant difference in p27 expression is only observed between homogeneous leukoplakia and erythroleukoplakia, but not with other types of non-homogeneous leukoplakia. Importantly, normal mucosa and erythroleukoplakia have similar numbers of p27-labelled cells. In this case, it is not necessary to compare the figures; however, we wish to emphasise the localisation pattern of p27 expression in erythroleukoplakia: immunopositivity occurs in the basal layer of the mucosa and in the cytoplasm of dysplastic cells, and therefore the figures become similar in both cases. It should be noted that in the superficial zones of the OL, the nuclear labelling with p27 disappears with keratinisation.

The concomitant reduction of p27 in the nuclei and the appearance of this protein in the cytoplasm suggest that p27 translocation from the nucleus to the cytoplasm, which was already very pronounced in the carcinomas we analysed, starts in this potentially malignant lesion. The weak expression of p27 antigen in the cytoplasm of erythroleukoplakia epithelial cells is explained by

the migration of macromolecules and ions between the nucleus and cytoplasm through nuclear pores (Ibarra et al., 2015); however, proteins are transported to ribosomes, the endoplasmic reticulum and the Golgi complex, where active protein metabolism occurs (Zheng et al., 2022).

As we assessed p27 expression in leukoplakia and carcinomas in three fields of view, our results and those of other researchers reflect expression of this antigen in a single histological section 4 μm thick, but not in the whole lesion volume, where the quantitative amount of p27 antigen is many fold higher than in these few fields of view. This should be taken into account in the future development of targeted tumour therapies in cell cultures, animal models and human tissue with any antigen. Our finding that carcinoma stage (T 1, 2, 3, 4) correlates very closely in descending order with p27 expression has important clinical and morphological implications. However, every investigator should be aware that T values do not reflect the extent/volume of malignancy, which is important for individual tumour targeted therapy. In molecular pathology and oral medicine, a clear distinction should be made between experimental and clinical studies on cell cycle proteins, as the results of cell culture and animal model studies may be unjustified and unsupported in practical oral medicine (Satoh et al., 2016; Vairaktaris et al., 2007).

We have not only analysed p27 expression in leukoplakia of different clinical variants, but also compared the results with a group of oral carcinomas. Oral squamous cell carcinoma was diagnosed in 40 % of patients at late stages (III and IV-a) with spread to lymph nodes. Information on p27 in oral carcinoma is controversial, as in the last two decades completely opposite results have emerged: some research groups have reported increased p27 protein (Hashmi et al., 2019; Mineta et al., 1999), while others have reported decreased expression of this antigen (Kudo et al., 1998; Shintani et al., 2002). The conflicting results may be explained by the different methods used to calculate p27 expression,

although the same antibody was used. In our study, p27 antigen-labelled cells were reduced 4.7-fold in oral carcinoma compared with erythroleukoplakia, seven-fold in homogeneous leukoplakia and 4.8-fold compared with healthy mucosa. However, a statistically significant difference in p27 was observed between oral carcinoma and healthy mucosa and between oral squamous cell carcinoma and all leukoplakia. In our oral carcinoma cases, intracytoplasmic p27 immunopositivity was more pronounced than in erythroleukoplakia. The location of p27 in the cytoplasm in malignancies is increasingly regarded as oncogenic by pathologists, but this fact is overlooked by some investigators (Ramasubramanian et al., 2013). We disagree, as p27 protein is ubiquitously demonstrated by standard immunohistochemistry as a protein with a molecular mass of 16.5 kDa, consists of 156 amino acids and has the same function. It is well known that there are 20 different amino acids in a cell, which can denature and lose their normal amino acid sequence. Disruption of the amino acid sequence is responsible for abnormalities in its function in the nucleus and cytoplasm.

For several years, the medical literature has reported that the cyclin-dependent kinase inhibitor p27 is a tumour suppressor, but it may also act as an oncogene in the cytoplasm (Sharma et al., 2016; Vallonthaiel et al., 2016). Our study demonstrates that p27 is gradually lost in oral carcinoma. We suggest that the gradual loss of p27 from the nucleus affects centrosome activity and distorts proper epithelial cell division. So p27 protein is a tumour promoter but a rather unstable oncogene. The variable expression of p27 protein in the region adjacent to the tumour and its centre indicates the dual nature of this antigen in tissues even a few millimetres away.

On the other hand, based on data from other researchers, we see that instead of p27 other proteins (p57, p63, p73) are diagnosed in malignant tumours. For example, p73 has a molecular mass of 77 kDa with 636 amino acids. So it is

possible that the amino acids rearrange to form other proteins. The p27 antigen has been termed a "substantially unstructured protein" or "enigmatic protein" by researchers, mainly due to advances in genomic analyses and a reassessment of the role of the gene encoding CDKN1B p27Kip1 in tumourigenesis (Bencivenga et al., 2017). Apparently, the biochemical structure of the p27 protein is fragile, and it is possible that these proteins turn into each other, as evidenced by numerous publications on the expression changes of p14, p16, p21, p27, p53, p73 and other antigens in the oral precancerous and malignant epithelium of squamous cell processes (Cema et al., 1998; Choi et al., 2003; Kresty et al., 2008; Sridharan et al., 2016; Visioli et al., 2012).

The present study demonstrates some alterations in the cytoplasm and nuclei of oral leukoplakia epithelial cells as well as mononuclear stromal cells during the malignisation process of predominantly non-homogeneous leukoplakia. In the future, the analysis of OL clinical data should be combined not only with immunohistochemical examinations, but also with findings of geneticists and electron microscopists on the progression of squamous epithelial dysplasia to malignancy and its treatment options, which could be implemented in the RSU laboratories.

The materials of all patients with clinically confirmed oral leukoplakia were used. Therefore, one of the limitations of the study is the small number of patients in the study group, which may reduce statistical significance. The relatively small number of patients could be due to the fact that patients with oral leukoplakia do not complain of oral changes and pain. Also, not all dentists are experienced in diagnosing OL, so not all patients are referred for consultation at the Institute of Dentistry Oral Medicine Centre. The number of patients in our study was significantly affected by the timing of the Covid-19 pandemic, when consultations at the Oral Medicine Centre were limited.

Conclusions

1 In the study group, oral leukoplakia was more common in men with a mean age of 57 years. The mean size of OL was 14 ± 3.7 mm. The most frequent site was the mucosa of the cheeks and lateral tongue, with severe dysplasia in 26 % of cases, which is also a risk factor for malignant transformation of OL.

2 VELscope fluorescence spectroscopy allowed the assessment and delineation of the altered tissue in both OL and carcinoma cases, thus improving the accuracy of biopsy site identification.

3 With increasing severity of the clinical form of oral leukoplakia, an increase in salivary total protein level in combination with a positive soluble CD44 test suggests early microenvironmental changes in the epithelium.

4 Our immunohistochemical study of CD 44 antigen demonstrated diversity of its amount and pattern in healthy, hyperplastic, dysplastic epithelium of different types of oral leukoplakia and malignant epithelium of squamous cell carcinoma. CD9 of exosomes demonstrated transport proteins in oral mucosa and helped to understand the movement of saliva and other fluids in healthy mucosa and its pathological lesions. The intra-cytoplasmatic expression of CD44 and CD9 antigens together with characteristic nuclear changes may be used as a predictive factor for potential malignant transformation of non-homogenous leukoplakia.

5 p27 glycoprotein showed suppressor and oncogenic properties in non-homogeneous OL and OSCCs, demonstrating the dual properties of this antigen with p27 restructuring dynamics from the nucleus to the cytoplasm. ThPOK is a low-informative marker in the study of malignant processes of oral leukoplakia.

6 Thick leukoplakia with severe dysplasia, translocation of CD44, CD9 and p27 proteins and elevation of soluble CD44 and total protein in saliva

characterise non-homogeneous oral leukoplakia as a high-risk potentially malignant oral neoplasm.

Proposals

1 In dental practices, we recommend the use of salivary CD44 and total protein tests for screening of oral malignancies and early diagnosis of recurrences without invasive methods.

2 We recommend VELscope fluorescence spectroscopy for the delineation of altered oral tissue borders in both oral leukoplakia and carcinoma to clarify biopsy or resection line sites.

3 Clinicians should detail all clinical information on both oral leukoplakia and oral carcinoma in forms 014/U-1 (Referral for pathohistological examination, P. Stradiņš CUS) and in the future also in the local databases of medical institutions and in the E-health system, which is very important for dynamic examinations, especially if they are performed in another hospital.

4 For pathologists to detect early signs of malignancy in oral leukoplakia, in addition to routine histological methods, we recommend immunohistochemical tests with CD44, CD9 and p27 antibodies to predict the development of altered tissues.

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

Internationally cited publications:

1 **Dzudzilo, M.,** Kleina, R., Čēma, I., Dabuzinskiene, A., Svirskis, Š., 2021, April. Expression and localisation of CD44 antigen as a prognostic factor of oral leukoplakia. *Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences.* 75, 2, 68–74.

2 Čēma, I., **Dzudzilo, M.,** Kleina, R., Franckeviča, I., Svirskis, Š., 2021, November. Correlation of soluble CD44 expression in saliva and CD44 protein in oral leukoplakia tissues. *Cancers*. 13, 22, 22 p., 5739; doi:10.3390/cancers13225739.

3 **Dzudzilo, M.,** Čēma, I., Kleina, R., Svirskis, Š., Selga, G., 2022. Characteristics of the dual nature of the p27 protein in oral leukoplakias and cancer. *Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences.* 76, 5/6, 665–673.

Oral presentations at international conferences:

1 **Dzudzilo, M.,** Čēma, I., Kleina, R., Franckeviča, I., Šmits, A. 2019. Pattern of CD44 Antigen Expression in Mucosal and Submucosal Structures in Case of Oral Leukoplakia. *RSU International Research Conference on Medical and Health Care Sciences, Knowledge for Use in Practice,* 1–3 April. Riga, Latvia.

2 **Dzudzilo, M.,** Kleina R., Čēma, I., 2020. CD44 antigen as an indicator of early structural changes in oral leukoplakia. *The 62nd International Scientific Conference of Daugavpils University*. May 28–29, 2020. Daugavpils, Latvia.

3 **Dzudzilo, M.,** Čēma, I., Kleina, R., Selga, G., Svirskis, Š., 2022. The role of expression of soluble CD44 in saliva and CD44 antigen in oral leukoplakia. *10th Congress of Baltic Association for Maxillofacial and Plastic Surgery and 16th Joint Symposium Riga-Rostock.* June 3, 2022. Riga, Latvia.

4 **Dzudzilo, M.,** Čēma, I., Kleina, R., Selga, G., 2023. Dysregulation of CD44 and p27 antigens in oral leukoplakia. *RSU Zinātnes nedēļa*. 27.–31.marts, 2023. Rīga, Latvija.

5 **Dzudzilo, M.,** Čēma, I., Kleina, R., 2023. Biomarkers indicating early malignancy in oral leukoplakia. *OPMDay Annual Congress.* August 30–31, 2023. Antwerp University, Belgium. **Best oral presentation award.**

Oral presentations at local conferences in Latvia:

1 **Dzudzilo, M.,** Čēma, I., Selga, G., 2016. VELscope lietojuma iespējas agrīnu malignitātes pazīmju diagnostikā mutes gļotādā. RSU Research Conference, 2016. Riga, Latvia.

2 **Dzudzilo, M.,** Čēma, I., Kleina, R., 2017. Pokemon – perspektīvs agrīna mutes vēža marķieris. RSU Scientific Conference, 2016. Riga, Latvia.

3 **Dzudzilo, M.,** Čēma, I., Kleina, R., 2021. Early detection of potentially malignant changes in oral leukoplakia using a saliva test. RSU Research week 2021: Knowledge for Use in Practice. March 24, 2021. Riga, Latvia.

4 **Dzudzilo, M.,** Čēma, I., Selga, G., Nazarovs, J., 2022. Biomarķieru pielietojuma iespējas agrīnu malignitātes pazīmju noteikšanai mutes leikoplakijās. Latvijas ārstu 9. kongress. 23. Septembris, 2022. Rīga, Latvija.

Theses at international conferences:

1 Kleina, R., **Dzudzilo, M.,** Čēma, I., 2017. *Variety of CD44 glycoprotein and immune cell reactions in oral squamous cell carcinoma and its possible precursors.* 9th Baltic morphology conference. 27–29 September, 2017. Tartu, Lithuania.

2 **Dzudzilo, M.,** Čēma, I., Kleina, R., Franckeviča, I., Šmits, A. 2019. Pattern of CD44 Antigen Expression in Mucosal and Submucosal Structures in Case of Oral Leukoplakia. RSU International Research Conference on Medical and Health Care Sciences, Knowledge for Use in Practice, 1–3 April. Riga, Latvia. 389.

3 Kleina, R., **Dzudzilo, M.,** Čēma, I., 2019. Th-POK Antigen Characteristics in Oral Leukoplakia. 10th Scientific Conference BALTIC MORPHOLOGY. October 24–25, 2019. Kaunas, Lithuania. 55, 264.

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5 **Dzudzilo, M.,** Kleina R., Čēma, I., 2020. CD44 antigen as an indicator of early structural changes in oral leukoplakia. The 62nd International Scientific Conference of Daugavpils University. May 28–29, 2020. Daugavpils, Latvia. 49.

6 **Dzudzilo, M.,** Čēma, I., Kleina, R., 2021. Expression of CD44 in potentially premalignant oral epithelial lesion – leukoplakia. 15th Biennial Congress European Association of Oral Medicine (EAOM). 2021. Porto, Portugal, 102–103.

7 **Dzudzilo, M.,** Čēma, I., Kleina, R., Selga, G., 2022. Characteristics of p27 protein diversity in oral leukoplakia and cancer. Oral Potentially Malignant Disorders (OPMD): state of the art and a roadmap for research. February 25, 2022. Brescia, Italy, 16.

8 **Dzudzilo, M.,** Čēma, I., Kleina, R., Selga, G., Svirskis, Š., 2022. The role of expression of soluble CD44 in saliva and CD44 antigen in oral leukoplakia. 10th Congress of Baltic Association for Maxillofacial and Plastic Surgery and 16th Joint Symposium Riga-Rostock. June 3, 2022. Riga, Latvia.

9 **Dzudzilo, M.,** Čēma, I., Kleina, R., Selga, G., 2023. Dysregulation of CD44 and p27 antigens in oral leukoplakia. RSU Zinātnes nedēļa. 27.–31.marts, 2023. Rīga, Latvija.

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Annexes

Annex 1

Patient information and consent form

Controller - SIA "Rīga Stradiņš University Institute of Dentistry"
Clinic of Oral Pathology
Reg. No 40003579713
Address.

_____
Name, surname

_____
Personal code

Consent to the processing of tissue data of a natural person

Please read the information below and tick whether you agree/disagree.

The Oral Pathology Clinic is developing a scientific study "Detection of Early Signs of Malignancy of Oral Leukoplakia in Oral Tissues and Saliva", which will study the expression of certain cancer stem cells also in healthy mucosal tissues.

We ask for your consent to use the tissue obtained during surgery for the purpose of the study. The tissue will be used anonymously in the study.

I am aware of my right to withdraw my consent at any time.

☐ **I agree**
☐ **I disagree**

Riga,

_____ 2016

Signature

Decision of the RSU Ethics Committee

Veidlapa Nr. E-9 (2)

RSU ĒTIKAS KOMITEJAS LĒMUMS NR. 3 / 18.08.2016.

Rīga, Dzirciema iela 16, LV-1007
Tel. 67061596

	Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1.	Profesors Olafs Brūvers	Dr.theo.	teologs
2.	Profesore Vija Stle	Dr.phil.	filozofs
3.	Asoc.prof. Santa Purviņa	Dr.med.	farmakologs
4.	Asoc.prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
5.	Profesore Regīna Kleina	Dr.med.	patalogs
6.	Profesors Guntars Pupelis	Dr.med.	ķirurgs
7.	Asoc.prof. Viesturs Liguts	Dr.med.	toksikologs
8.	Docente Iveta Jankovska	Dr.med.	
9.	Docents Kristaps Circenis	Dr.med.	

Pieteikuma iesniedzējs: **Dr. Madara Dzudzilo**
Medicīnas fakultāte, doktorantūra

Pētījuma nosaukums: „ Agrīna malignitātes pazīmju diagnostika siekalās un mutes vēžģraudes audos"

Iesniegšanas datums: 15.08.2016.

Pētījuma protokols: Izskatot iesniegtos pētījuma dokumentus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts, bez kāda apdraudējuma veselībai, drošībai un dzīvībai, pacientus iedalot 3 pētījuma grupās – pirmajā pētījuma grupā piedalīsies pacienti, kas ieradušies konsultācijai SI Orālās Patoloģijas klīnikā un devuši savu piekrišanu pētījumā. Pacients tiks iekļauts pētījumā, kam klīniski diagnosticēts priekšvēža saslimšana mutes dobumā. Šiem pacientiem tiks ņemta biopsija, morfoloģiski pierādīta displāzija vai vēža klātbūtne un veikti imūnhistoķīmiskie pētījumi par biomarķieru ekspresiju. Otrajā pētījuma grupa analizēs morfoloģiskos arhīva datus ar jau pierādītu plakanšūnu vēzi un veiks imūnhistoķīmisko biomarķieru ekspresijas analīzi. Trešajā pētījuma grupā iekļauskontroles grupas pacientus, konsultētus SI Orālās Patoloģijas klīnikā. Pēc iegūto datu apstrādes un analīzes, tiek sniegti priekšlikumi. Personu (pacientu, dalībnieku) datu aizsardzība, brīvprātīga informēta piekrišana piedalīties pētījumā un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst pētījuma ētikas prasībām.

Izskaidrošanas formulārs: ir

Piekrišana piedalīties pētījumā: ir

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers Tituls: Dr. miss., prof.

Paraksts

Ētikas komitejas sēdes datums:18.08.2016.