Molecular Insights in Oral Leukoplakia

Epigenetic, Immunological, and Proteomic Perspectives

Divya Ganesh

Department of Oral medicine and Pathology

Institute of Odontology

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: Molecular changes occurring in a cell, showing intracellular Toll-like receptors present on the surface of endosomes on the left, epigenetic regulation of EZH2 in the nucleus on the middle and proteins involved in the malignant transformation process of leukoplakia on the right side. Created in BioRender. Ganesh, D. (2025) https://BioRender.com/ls5vomp

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divya.ganesh@gu.se

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வாய்நாடி வாய்ப்பச் செயல்.

- திருவள்ளுவர்
300 BCE to 500 CE

Meaning:
"Examine the disease, find out its cause, study the remedy, and then apply it at the right time."

To my loveable family

Molecular Insights in Oral Leukoplakia- Epigenetic, Immunological and Proteomic Perspective

Divya Ganesh

Department of Oral Medicine and Pathology, Institution of Odontology, Sahlgrenska Academy, University of Gothenburg, Sweden, 2025.

ABSTRACT

Oral leukoplakia, a potentially malignant oral disorder, with a risk of transformation into oral squamous cell carcinoma (OSCC). This thesis investigates the molecular, immunological, and proteomic alterations in leukoplakia, aiming to identify early cancer transformation markers.

Study I examined the expression of the epigenetic regulator EZH2 in leukoplakia. Increased nuclear EZH2 expression in transforming leukoplakia, correlated directly with CD3 positive- and CD8 positive T-cell infiltration in the epithelium, suggesting an immune response and epithelial dysregulation.
Study II explored the localization and expression of Toll-like receptors (TLR3, TLR7, TLR8, TLR9) in leukoplakia. Nuclear translocation of TLR7 was significantly associated with dysplasia, while reduced cytoplasmic expression of TLR8 and TLR9 might play a role in immune dysregulation during transformation.
Study III assessed the expression of intracellular TLRs in oral epithelial cell lines (DOK, CaLH3, SCC25). TLR3 and TLR9 expression were inducible in DOK and CaLH3 but absent in SCC25. Stimulation with TLR agonists led to downregulation of TRIF. The TLR7 agonist imiquimod inhibited DOK cell proliferation, suggesting its therapeutic potential in early-stage oral lesions.
Study IV employed proteomic profiling of leukoplakia not transforming and transforming to OSCC and OSCC tissues. Proteins related to cytoplasmic trafficking, matrix remodeling, and immune pathways were differentially expressed during progression from leukoplakia to OSCC. Notably, nuclear expression of EEF1D and cytoplasmic expression of Perlecan was evident in OSCC, suggesting subcellular shifts might be responsible for transformation.

Collectively, these studies highlight epigenetic reprogramming, immune modulation, and proteomic remodeling from leukoplakia that progress into OSCC. In the future, integrating transcriptomic, proteomic, and spatial analyses may enable early detection, enhance diagnostic and treatment precision in patients with leukoplakia.

Keywords: EZH2, oral squamous cell carcinoma, potentially malignant oral disorders, T cells, Toll-like receptors

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SAMMANFATTNING PÅ SVENSKA

Oral leukoplaki är en potentiellt malign oral slemhinnesjukdom med en risk för transformation till oral skivepitelcancer (OSCC). Denna avhandling undersöker epigenetiska, immunologiska och proteomiska förändringar i leukoplakier- med syfte att identifiera tidiga markörer för malign transformation vilket kan möjliggöra bättre riskbedömning och behandling.

Studie I analyserade uttrycket av den epigenetiska regulatorn EZH2 i leukoplakier. Förhöjt nukleärt EZH2-uttryck i cancertransformerande leukoplakier korrelerade direkt med infiltration av CD3 positiva- och CD8 positiva T-celler i epitelet, vilket indikerar en immunaktivering och epitelial dysreglering.

Studie II undersökte uttryck och lokalisation av Toll-like receptorer (TLR3, TLR7, TLR8, TLR9) i leukoplaki. Nukleär translokation av TLR7 var signifikant associerad med dysplasi, medan reducerat cytoplasmiskt uttryck av TLR8 och TLR9 kan bidra till immunologisk dysreglering under transformation.

Studie III utvärderade intracellulärt TLR-uttryck i orala epitelceller (DOK, CaLH3, SCC25). TLR3 och TLR9 var inducerbara i DOK och CaLH3 men frånvarande i SCC25. Stimulering med TLR-agonister ledde till nedreglering av TRIF. Imiquimod hämmade proliferation i DOK-celler, vilket indikerar terapeutisk potential av imiquimod i leukoplakier.

Studie IV användes proteomisk profilering av icke-transformerande leukoplakier, transformerande leukoplakier och OSCC. Proteiner kopplade till matrixremodellering, lipidmetabolism och immunvägar uppvisade förändrat uttryck i leukoplakier. Framträdande var nukleärt uttryck av EEF1D och cytoplasmatiskt uttryck av Perlekan i OSCC, vilket tyder på att subcellulära förskjutningar kan bidra till transformation.

Sammanfattningsvis belyser dessa studier epigenetisk omprogrammering, immunmodulering och proteomisk omstrukturering i de leukoplakier som övergår i OSCC. I framtiden kan integrering av transkriptomiska, proteomiska och spatiala analyser kan möjliggöra bättre riskbedömning och mer precisionsinriktad diagnostik och behandling av patienter med leukoplaki.

LIST OF PAPERS

This thesis is based on the following studies,

Ganesh D, Dafar A, Niklasson J, Sandberg I, Braz-Silva P, Sapkota D, Öhman J, Giglio D, Hasséus B. EZH2 Expression Correlates With T-Cell Infiltration in Oral Leukoplakia and Predicts Cancer Transformation. Anticancer Research 2023 Apr;43(4):1533-1542.

Ganesh D, Sundberg J, Dafar A, Kjeller G, Sapkota D, Öhman J, Giglio D, Hasséus B. Expression of intracellular Toll-like receptors in leukoplakia and oral squamous cell carcinoma. (Accepted in Acta Odontologica Scandinavica)

Ganesh D, Öhman J, Sapkota D, Torinsson Naluai Å, Giglio D, Hasséus B. Toll-like receptors signaling in oral premalignant and cancer cell lines. (Manuscript)

Giglio D, **Ganesh D**, Bhattarai BP, Soland TM, Sundberg J, Thorsell A, Öhman J, Sapkota D, Hasséus B. Biomarkers Correlating with the Development of Oral Squamous Cell Carcinoma from Leukoplakia. (Under review in Scientific Reports)

CONTENTS

ABBREVIATIONS

OSCC	Oral Squamous Cell Carcinoma
OPMDs	Oral Potentially Malignant Disorders
WHO	World Health Organisation
TLRs	Toll-like receptors
DAMPs	Damage-associated molecular patterns
PAMPs	Pathogen-associated molecular patterns
IFN	Type I interferon
APC	Antigen-presenting cells
T regs	Regulatory T cells
EZH2	Enhancer of zeste homolog 2
NPC	Nuclear pore complex
OL-non/ leuko-nonca	Non-transforming leukoplakia
OL-ca/ leuko-ca	Transforming leukoplakia

OL-dys	Hyperkeratosis with dysplasia
OL-no	Benign hyperkeratosis
SCC25	Squamous cell carcinoma 25
CalH3	Cellosaurus cell line
DOK	Dysplastic Oral Keratinocytes
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
Perlecan	Basement membrane-specific heparan sulfate proteoglycan
ApoE	Apolipoprotein E
EEF1D	Eukaryotic elongation factor1-delta
ABCF2	ATP-binding cassette sub-family F member 2
PGAM5	Phosphoglycerate mutase 5
SD-leuko-ca	OSCC developed 5-26 months after OL diagnosis (short duration-SD)

LD-leuko-ca	OSCC developed 32-54 months after OL diagnosis (long duration-LD)
miRNA	Micro RNA

DEFINITIONS IN SHORT

Oral potentially malignant disorders	*A heterogenous group of clinically defined conditions associated with a variable risk of progression to oral squamous carcinoma*
Oral leukoplakia	*A predominantly white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer (WHO 2007)*
Epigenetics	*The inheritance of DNA activity that does not depend on the naked DNA sequence.*

1 INTRODUCTION

1.1 ORAL POTENTIALLY MALIGNANT DISORDERS

Oral potentially malignant disorders (OPMDs) are defined as ¨a heterogenous group of clinically defined conditions associated with a variable risk of progression to oral squamous carcinoma¨ [1]. OPMDs are confined as a single category by World Health Organization (WHO) (2007), it refers to a spectrum of lesions and conditions with varying risk of progressing to cancers of the oral cavity [2]. OPMDs present with diverse clinical appearances, ranging from white, red, or mixed red-white lesions to surface changes such as smooth, corrugated, verrucous, granular, or atrophic patterns, and they can vary considerably in size [3, 4]. The 2020 WHO Collaborating Centre consensus replaced the older terminology of "lesions/conditions" with the broader term "disorders" in the definition of OPMDs. It also expanded the classification to include lichenoid lesions and Oral graft versus host disease [5].

Table 1:WHO 2022 classification of oral potentially malignant disorders [1].

Leukoplakia
Proliferative verrucous leukoplakia
Erythroleukoplakia
Erythroplakia
Submucous fibrosis
Oral lichen planus
Palatal lesions in reverse smokers
Smokeless tobacco keratosis
Lupus erythematosus
Oral lichenoid lesions
Oral graft versus host disease
Familial cancer syndromes including Fanconi anaemia, cowden syndrome, dyskeratosis congenita, bloom syndrome, LI-Fraumeni syndrome, xeroderma pigmentosum and ataxia-telangiectasia

According to WHO 2022 classification, encompass a spectrum of oral potentially malignant disorders, it is listed in Table 1 [1]. The WHO 2022 classification, actinic keratosis was removed from the list of

OPMDs [1]. In this thesis we will discuss oral leukoplakia and malignant transformation.

1.2 ORAL LEUKOPLAKIA: DEFINITION AND CLINICAL SIGNIFICANCE

Oral leukoplakia was defined by the WHO in 2007 as "*A predominantly white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer*", whereas 2020 WHO group did not change this definition [5]. Leukoplakia usually appears as persistent white patches or plaques that cannot be removed and is often asymptomatic but may occasionally cause burning sensation or discomfort (Fig. 1) [5].

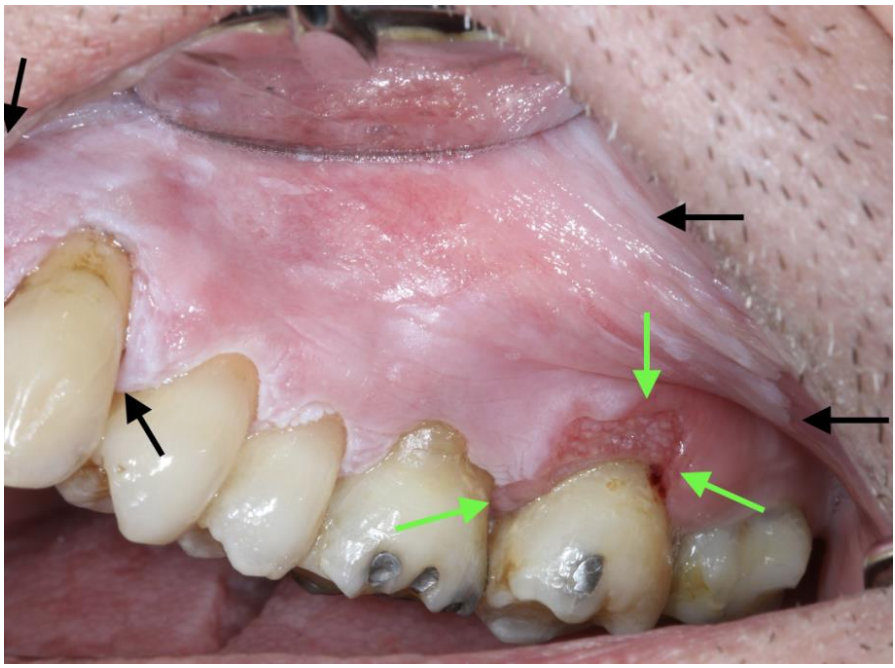

Figure 1: Clinical image of patient with leukoplakia indicated with black arrows, green arrows indicating leukoplakia transformed into oral squamous cell carcinoma (Photo by Dr. Jenny Öhman).

Leukoplakia is categorized into non-homogeneous and homogeneous types. Homogeneous leukoplakia appears as flat, uniformly white plaques with smooth or may exhibit shallow cracks, while non-homogeneous leukoplakia includes verrucous, nodular, or speckled (red or white) variants, which carry a higher risk of malignant transformation [5].

1.2.1 ETIOLOGY

The etiology of leukoplakia is multifactorial, though many cases remain idiopathic [6]. Tobacco use, in both smoked and smokeless forms such as areca (betel) nut and snuff, represents the primary risk factor, while alcohol consumption and viral infections also contribute to disease development [7, 8]. Chronic candidiasis could be associated with leukoplakia, although conclusive evidence supporting a causal role is still lacking [9].

1.2.2 EPIDEMIOLOGY

A systematic review on global prevalence of leukoplakia revealed around 1.4%, with pooled rates varying between 2.2% in population-based studies**,** 1.4% in clinic-based cohorts, and 9.1% in high-risk groups. Prevalence differed between geographical regions, with figures ranging from 0.3% to 11.7%. Incidence is higher in men, older individuals (>60 years), smokers, and alcohol users [10].

1.2.3 DIAGNOSIS

Diagnosing leukoplakia poses significant clinical challenges. The clinical appearance can range from small, homogeneous white plaques to large, non-homogeneous, speckled, or verrucous lesions, making it difficult to distinguish leukoplakia from other white oral lesions such as lichen planus, candidiasis, or frictional keratosis [5]. Thus, a tentative leukoplakia diagnosis can only be set when other diseases with similar clinical presentation have been excluded [7].

The gold standard for diagnosing leukoplakia is clinical diagnosis with histopathological confirmation with a tissue biopsy [5]. Excisional

biopsy is generally performed for small lesions, while incisional biopsy is preferred for larger ones [7].

Other diagnostic approaches such as toluidine blue staining, exfoliative cytology, analysis of salivary biomarkers, and optical imaging tools have been explored for non-invasive diagnosis [11]. A review on light-based devices reported that autofluorescence showed wide variability in sensitivity (33–100%) and specificity (12–88.6%). Fluorescent probes such as 5-aminolevulinic acid demonstrated higher accuracy (sensitivity 90–100%, specificity 51.3–96%) [12]. Larger studies on oral exfoliative cytology report its role as limited in diagnosing leukoplakia, occasionally detect candidiasis and in few cases, the Papanicolaou classification may suggest malignancy; however, biopsy remains the gold standard for definitive diagnosis [13].

1.2.4 HISTOPATHOLOGICAL FEATURES OF LEUKOPLAKIA

Leukoplakia is a clinical diagnosis and has no histopathologic counterpart, although histopathological examination is necessary to confirm the clinical diagnosis by excluding other possible diagnosis [5]. Presence of oral epithelial dysplasia in tissue specimens is important since higher grades of dysplasia correlates with risk of cancer transformation [14]. However, a tissue specimen can also reveal a benign hyperkeratosis without any signs of oral epithelial dysplasia [5].

The most common histopathological feature observed is a epithelial keratinization (ortho- or para-) and other features may vary depending on the type of leukoplakia [15, 16]. In leukoplakia, histopathological evaluation reveals either absence or presence of epithelial dysplasia, which is graded according to the cytological and architectural changes. WHO has recently established criteria for architectural and cytological features observed in oral epithelial dysplasia (Table 2) [17].

Table 2: Features of oral epithelial dysplasia using cytological and architectural changes adapted from WHO classification of head and neck tumors [17].

Architectural features	Cytological features
Keratin pearls within rete ridges	Nuclear size variation
Reduced intercellular cohesion	Cell size variation
Generalized premature keratinization	Cell shape variation
Increased mitotic activity (reclassified as cytological)	Nuclear shape variation
Mitotic figures higher in the epithelium	Elevated nuclear to cytoplasmic ratio
Drop-shaped rete ridges	Atypical mitosis
Irregular epithelial stratification	Enlarged or prominent nucleoli
Loss of polarity in basal cells	Nuclear hyperchromasia

New additions (2022):	
Multiple patterns of dysplasia within the same lesion	Single-cell keratinization
Clustering or nesting of basal cells	Apoptotic mitosis
Extension of atypical changes into minor gland ducts	Increased nuclear size
Well-demarcated margin of dysplastic change	
Altered keratin patterns depending on oral sub-site	
Multifocal or discontinuous lesions	
Expanded proliferative compartment	
Verrucous or papillary epithelial growth	

In the histopathological report presence of oral epithelial dysplasia is graded as mild, moderate and severe. Mild oral epithelial dysplasia is characterized by being limited to the basal and parabasal layers and with minimal cytological atypia and architectural disturbance (Fig. 2A). Moderate oral epithelial dysplasia extends into the middle third of the epithelium and display an increased nuclear pleomorphism and mitotic activity (Fig. 2B). In severe oral epithelial dysplasia affects over two-thirds of the epithelial thickness and there is a marked cytological atypia, marked pleomorphism abnormal mitoses, and architectural disturbance (Fig. 2C) [1, 18]. Recently, a two-tier dysplasia grading system has been proposed as the better system [19]. An advantage with such a system is a higher interobserver agreement [19]. From a clinical perspective it would also facilitate malignant transformation risk assessment since leukoplakia with low-grade dysplasia are less likely to progress to cancer than lesions with high grade dysplasia [20].

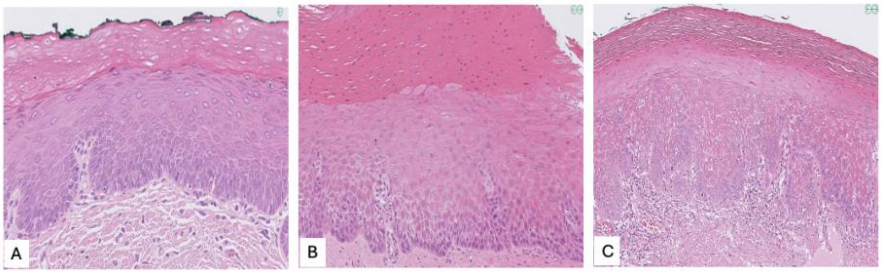

Figure 2: Histopathological features of oral epithelial dysplasia are graded into A. mild dysplasia, B. moderate dysplasia and C. severe dysplasia

1.2.5 TREATMENT

Treatment options for leukoplakia include both surgical and non-surgical approaches. The choice of treatment depends on lesion size, histopathological findings, risk factors, and patient preference.

Management starts with eliminating risk factors such as tobacco, alcohol, and candida infection [21]. If feasible, excision and subsequent histopathological analysis is recommended [7]. Non-dysplastic, low-risk lesions are generally monitored, whereas dysplastic or high-risk lesions are surgically treated using scalpel excision, laser excision and laser ablation is often preferred for its minimally invasive nature[21]. Photodynamic therapy with 5-aminolevulinic acid has shown promising results in leukoplakia with mild or moderate dysplasia [22].

Adjunctive chemoprevention with antioxidants (lycopene, vitamins A and curcumin) may reduce lesion size, though outcomes remain inconsistent, with lycopene showing the strongest evidence, however a recent study on imiquimod showed 50% reduction in size of leukoplakia lesions in two-third of the cases [21, 23]. As recurrence is frequent and malignant transformation unpredictable, individualized treatment and long-term surveillance are essential[24].

A systematic review on leukoplakia shows a recurrence rate of about 22% [25]. Recurrence is more common in non-homogeneous lesions, those in the retromolar area or present at multiple sites, older patients, females, or betel quid users [25]. Laser-based approaches, especially when combining excision with vaporization, were associated with the

lowest recurrence rates [25]. Our previous study reported that recurrence of leukoplakia after surgical removal reached 49% within 5 years, mainly associated with non-homogeneous type and snuff use [26].

1.2.6 RISK OF MALIGNANT TRANSFORMATION

A meta-analysis of 52 studies including over 41,231 leukoplakia cases reported a pooled malignant transformation rate of 6.6%. Transformation risk was higher in smokers with larger non-homogeneous lesions**,** sites such as tongue lateral border and epithelial dysplasia [20]. While histopathological grading of epithelial dysplasia remains the gold standard, it suffers from subjectivity, inter-observer variability, and sampling errors, particularly when lesions are large or multifocal [3].

Leukoplakia with dysplasia showed a higher malignant transformation risk in comparison with those without dysplasia, and the risk rose with increasing severity [21]. Hazard ratios were 4.9 for mild, 6.0 for moderate, and 15.8 for severe dysplasia. Five-year cancer risk estimates were 2.2% for non-dysplastic lesions, 11.9% for mild, 8.7% for moderate, and 32.2% for severe dysplasia. Notably, nearly 40% of cancers still developed from lesions without histological evidence of dysplasia [27].

1.3 ORAL SQUAMOUS CELL CARCINOMA

1.3.1 ETIOLOGY

Oral squamous cell carcinoma (OSCC) develops from genetic changes, epigenetic alterations and dysregulation in the keratinocytes of the oral squamous epithelium [28]. OSCC may develop from pre-existing OPMDs or can also develop spontaneously [29]. In addition to OPMDs, OSCC has been strongly associated with risk factors such as tobacco and alcohol use, human papillomavirus infection, and nutritional deficiencies [28].

1.3.2 EPIDEMIOLOGY

OSCC is the most common malignancy of the oral cavity, contributing for over 90% of all oral tumors [30]. International Agency for Research on Cancer (IARC) states that, cancers of lip and oral cavity affected about 377,713 people in 2020, with 177,757 deaths, ranking 18th globally in incidence [31]. Oral cancer occurs more often in men, with about 6% of cases found in those under 45 years [30]. Other oral cavity tumors, such as salivary gland tumors, hematologic tumors, bone tumors, mesenchymal tumors, odontogenic tumors are relatively rare [32]. Despite the accessibility of the oral cavity for examination, many cases are detected late due to misdiagnosis or patient delay.

According to NORDCAN (2016-2020), the age-standardized incidence rate (Nordic standard) for oral cavity cancer across the Nordic countries was 5.2 per 100,000 men and 3.7 per 100,000 women, with roughly 816 new male cases and 642 new female cases annually (2019-2023) [33]. Focusing on Finland and Sweden, combined data through 2019 show that for oral cavity and pharyngeal cancers among men, the incidence was about 2.4 per 100,000 in Finland and 2.1 per 100,000 in Sweden. Among women, the corresponding rates were 1.5 per 100,000 (Finland) and 1.4 per 100,000 (Sweden) [34].

1.3.3 DIAGNOSIS

OSCC usually presents as an aberrant growth in the oral cavity, often ulcerated, and with induration upon palpation [35, 36]. On clinical suspicion of OSCC the gold standard is tissue biopsy and upon confirmation of a cancer diagnosis immediate further examinations should be initiated by the clinician.

1.3.4 HISTOPATHOLOGICAL FEATURES OF ORAL SQUAMOUS CELL CARCINOMA

OSCC is characterized histopathological by the invasion of dysplastic squamous epithelium through the basement membrane into underlying connective tissue. Key features include sheets, nests, or islands of malignant squamous cells with eosinophilic cytoplasm, increased

mitotic activity, hyperchromatic and pleomorphic nuclei, intercellular bridges and individual cell keratinization. Well-differentiated tumors often show prominent keratin pearl formation, while poorly differentiated tumors display more pronounced cellular and nuclear atypia with less keratinization. Depth of invasion, lymphovascular or perineural invasion, and grading (well, moderately, or poorly differentiated) are also crucial histopathological parameters in OSCC [1, 37].

1.3.5 STAGING AND GRADING OF ORAL SQUAMOUS CELL CARCINOMA

Staging and grading are essential in OSCC management as they determine risk classification and guide treatment planning [38]. The AJCC/UICC Tumor, Node, Metastasis staging system, first introduced in 1960s, updated in 1970s and most recently updated in 2017 (8th edition), remains the global standard [39]. Primary tumors are classified from Tis (in situ) to T4 based on size and local invasion, while lymph node involvement ranges from N0 (no metastasis) to N3 (nodes >6 cm or bilateral spread). Metastasis is graded as M0 (absent) or M1 (distant spread) [40]. Together, these parameters form the basis of OSCC staging (0–IV).

1.3.6 TREATMENT AND PROGNOSIS

Standard OSCC management modalities include surgery remains as a standard approach, radiotherapy, chemotherapy in combination or alone remains adjuvant for patients with high-risk OSCC and also recently immunotherapy is in clinical trials but still survival rates remain poor often due to late detection and treatment resistance [41]. Presently, Food and Drug Administration in the United States approved drugs for OSCC include chemotherapeutics (docetaxel, cisplatin, and fluorouracil), EGFR inhibitors (cetuximab) and immune checkpoint inhibitors (pembrolizumab) [41, 42]. However, response to EGFR- and PD-1 targeted therapies remain limited, highlighting the need for precision approaches and novel drug delivery strategies [43].

The five-year survival rate for oral cavity, tongue, and oropharyngeal cancers averages around 50% across several countries [30].

1.4 HALLMARKS OF CANCER

The concept of "*Hallmarks of Cancer*", were first introduced by Hanahan and Weinberg (2000, 2011), describes the functional capabilities acquired by cells during malignant transformation. The hallmarks include sustaining proliferative signaling, enabling replicative immortality, evading growth suppressors, activating invasion & metastasis, resisting cell death and inducing/accessing vasculature [44]. In 2011, four additional characteristics were introduced: deregulating cellular metabolism, genome instability & mutation, avoiding immune destruction and tumor- promoting inflammation [45].

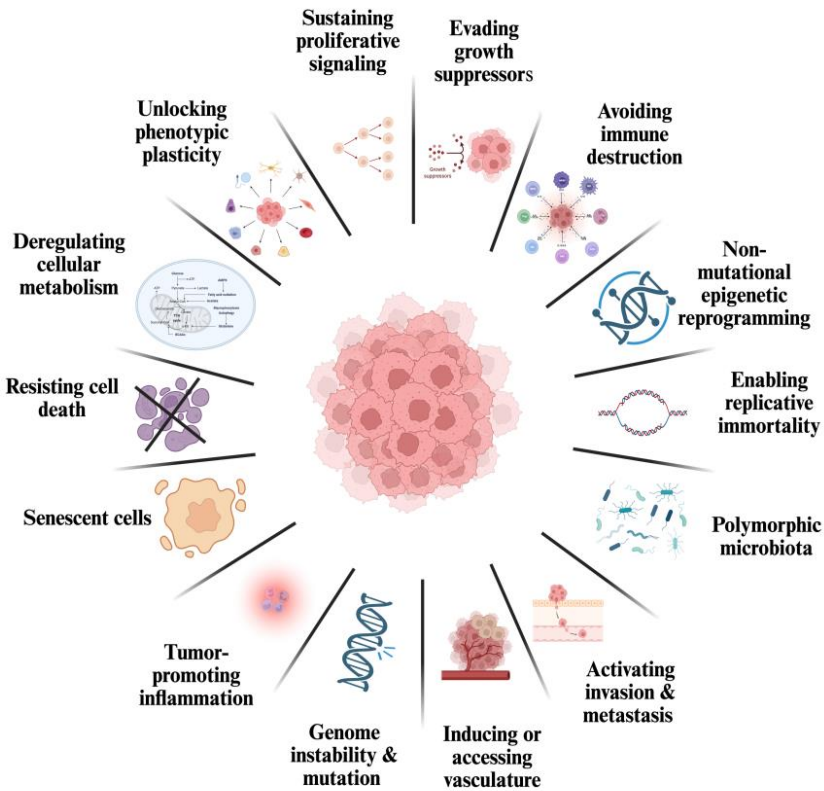

Figure 3: Hallmarks of cancer. Image was adapted from Frontiers in Immunology by Hanahan D [46]. Created in BioRender. Ganesh, D. (2025) https://BioRender.com/bc44ptf

Recent updates highlight additional emerging features such as unlocking phenotypic plasticity, senescent cells, non-mutational epigenetic reprogramming, and polymorphic microbiota are reflecting new insights into tumor heterogeneity and progression, as shown in (Fig. 3) [46].Collectively, these hallmarks emphasize that cancer is not only a disease of genetic mutations but also of altered signaling, disrupted differentiation, microenvironmental interactions, and immune evasion. Recent reviews have summarized biomarkers associated with the presence of hallmarks of cancer in leukoplakia and OSCC (Table 3) [47, 48].

Table 3:Comparison of hallmarks of cancer[46] in leukoplakia and OSCC. Adapted from Gonzales-Ruiz et al [47] and Jagadesan et al [48].

Hallmarks of Cancer[46]	Leukoplakia [47]	OSCC [48]
Sustaining proliferative signaling	Overexpression of EGFR, Ki-67, and Cyclin D1	EGFR overexpression Cyclin D1 are stimulated
Evading growth suppressors	Overexpression of p53, loss of pRb.	Mutations in p53, Loss of Rb checkpoint pathways
Resisting cell death	Disruption in apoptotic pathways	Dysregulation of apoptosis
Enabling replicative immortality	Telomerase activity not investigated	Increased telomerase activity
Inducing angiogenesis	Lack of evidence for angiogenesis in leukoplakia.	VEGF and FGF family members are commonly upregulated.
Activating invasion and metastasis	Altered adhesion markers (E-cadherin, β-catenin, Twist)	Evidence of epithelial–mesenchymal transition, exhibits low expression of E-cadherin
Deregulating cellular metabolism	Higher expression of LDH	Warburg effect, characterized by aerobic glycolysis
Avoiding immune destruction	No direct systematic evidence reported	Melanoma inhibitory activity (MIA), MIA2 are frequently expressed
Genome instability and mutation	3p and 11q are Amplified results in increased expression of CCND1	Mutations HRAS, CASP8, TP53, NOTCH 1, CDKN2A
Tumor-promoting inflammation	Elevated levels of IL-1β, IL-6, and TNF-α.	Cancer cells secrete IL-6, IL-8 develop drug resistance

Genetic alterations play a central role in the pathogenesis of cancer. Key mechanisms include altered chromosome numbers, copy number changes, chromosomal instability, loss of heterozygosity, telomere dysfunction, defective DNA repair, and deregulated cell-cycle checkpoints.

Most common genetic changes in OSCC are tumor suppressor genes such as p53, APC and Ras is an oncogene, MYC is a proto-oncogene and regulatory genes such as EIF3E and GSTM1 [49]. Amplification of 11q13 and cyclin D1 overexpression are linked to aggressive tumor [50], while TGF-α overexpression in early OSCC promotes proliferation and angiogenesis. NOTCH1 mutations are also implicated, though their precise role remains unclear [49, 51]. Inactivation of p16, often observed early, is one of the first tumor suppressor changes in OSCC [52]. KRAS mutations, strongly associated with poor differentiation, advanced stage, lymphovascular invasion, and metastasis, correlate with Cyclin D1, Bcl2, and Ki-67 expression, suggesting value as a prognostic marker [53].

The epithelial-mesenchymal transition, marked by increased N-cadherin, vimentin and decreased E-cadherin, facilitates invasion and metastasis, though low expression levels are associated with poor prognosis [54, 55]. Environmental risk factors such as tobacco and alcohol strongly correlate with these molecular changes [56]. Clinically, OSCC often arises from OPMDs such as erythroplakia, oral submucous fibrosis, leukoplakia and proliferative verrucous leukoplakia [57]. Despite therapeutic advances, patients with advanced OSCC frequently face poor survival outcomes [58].

Salivary microbiota studies show distinct separation of leukoplakia and OSCC from healthy controls by principal component analysis. Firmicutes dominated all groups but declined from healthy controls (73%) to leukoplakia (66.2%) and OSCC (60.3%), while Bacteroidetes and TM7 increased [59]. Streptococcus was most abundant in healthy controls but reduced in leukoplakia/OSCC, whereas Haemophilus was enriched in leukoplakia and Bacillus in OSCC, indicating microbial shifts linked to disease progression [59].

Leukoplakia samples showed higher microbial richness than controls, but diversity declined from OPMDs to carcinoma [60]. Streptococcus pneumoniae and Pseudomonas fluorescens were predominant in leukoplakia [60], while OSCC was enriched in periodontal pathogens such as Fusobacterium nucleatum, Prevotella intermedia and Porphyromonas gingivalis [61]. Increased levels of Capnocytophaga gingivalis, Lactobacillus, Prevotella melanogenica, and Streptococcus mitis may serve as diagnostic markers [62, 63].

1.5 THE IMMUNE SYSTEM

The immune system constitutes an intricate network of cellular and molecular components that operate in coordination to recognize, neutralize, and eliminate foreign antigens such as microbes, viruses, toxins, and dysplastic or malignant cells. In addition to physical and chemical barriers, immunity can be broadly divided into three main defense strategies: physiologic and anatomic barriers, innate and adaptive immunity [64].

1.5.1 ANATOMIC AND PHYSIOLOGIC BARRIERS

Anatomic and physiological barriers serve as the body's first line of protection against infectious agents**.** This includes skin and mucosa, effective clearance by mucociliary, acidic gastric pH, and antimicrobial enzymes such as lysozyme present in saliva, tears, and other secretions. The importance of these barriers is evident in individuals with severe burns or ciliary dysfunction, who show markedly increased susceptibility to infections [64].

1.5.2 INNATE IMMUNITY

The innate immune system is the body's primary protection against abnormal cells and infections [65]. It is present from birth and responds rapidly and short-term response that are non-specific to pathogens, recognizing general danger signals usually referred to as pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) rather than specific antigens [66]. Unlike the adaptive immune system, it does not have an immunological memory, but there

is an antigen-independent immune memory via epigenetic reprogramming [67, 68].

The innate immune system is further classified in a cellular and humoral part [64], The cellular immunity comprises of various hematopoietic cells such as neutrophils, dendritic cells, basophils, eosinophils, natural killer T- and B- cells, macrophages, mast cells and natural killer (NK) cells. In addition to these, non-hematopoietic cells like epithelial cells found in the skin, gastrointestinal lining and respiratory tract also play a crucial role, The humoral immunity of the innate immune system includes a wide array of molecules such as antibodies, lipopolysaccharide-binding protein, complement proteins, C-reactive protein, mannose-binding lectin, antimicrobial peptides and other acute-phase reactants [64].

DAMPs or PAMPs are recognized by pattern recognition receptors which include Toll-like receptors (TLRs), AIM2-like receptors, RIG-I-like receptors , NOD-like receptors, C-type lectin receptors, and other cytosolic DNA sensors [69]. Among these immune regulators, Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1) functions as an inhibitory pattern recognition receptor, targeting LAIR1 offers a potential therapeutic strategy to regulate immune activity and overcome immune suppression in cancer [70].

When activated by DAMPs, TLR receptors initiate downstream pathways such as NF-κB, inflammasome, and type I interferon (IFN) signaling, resulting in the release of pro-inflammatory cytokines, chemokines, and antiviral mediators [71]. This innate response not only shapes the inflammatory microenvironment but also promotes adaptive immunity, including the activation of CD8 positive T cell responses against tumor-associated antigens [27].

The innate immune system initiates and shapes the adaptive immune system by recognizing and responding to invading pathogens. Dendritic cells are antigen-presenting cells (APCs), that engulf both self- and foreign antigens and continuously migrating to lymphoid tissues where they present, in the context of major histocompatibility complex (MHC) molecules, processed antigens to naive T cells. This crosstalk provides the crucial bridge from immediate, non-specific defense to long-lasting,

highly specific immunity [72]. In the skin and mucosal linings, dendritic cells are known as Langerhans cells, and they provide the capacity for early detection of external threats.

1.5.3 ADAPTIVE IMMUNITY

The adaptive immune system develops over several days-weeks, offering precision and an immunological memory that enables rapid and effective responses upon re-exposure to the same pathogen [68, 73].

Further, the adaptive immune system consists of a cellular and humoral branch [64]. Cell-mediated immunity is driven by T cells. CD4 positive helper T cells coordinate immune functions by releasing cytokines that stimulate various immune cells, whereas CD8 positive cytotoxic T cells eliminates infected or transformed cells directly by killing. B cells are lymphocytes that recognize specific antigens via the B cell receptor and function as APC. Upon activation, they proliferate and interact with helper T cells to activate humoral response. Humoral immunity is mediated by B cells. Once activated, B cells mature into plasma cells that secrete antibodies capable of neutralizing pathogens, promoting their removal, and amplifying innate immune processes like phagocytosis and complement activation [64].

1.5.4 CANCER IMMUNOEDITING

Cancer immunoediting explains how the immune system can both inhibit and support tumor progression, a “double-edged sword” in cancer biology[74]. It encompasses three interconnected phases elimination, equilibrium, and escape [75]. During the elimination phase, both innate and adaptive immune cells recognize tumor-associated antigens and destroy transformed cells through dendritic cells, stimulated NK, NKT cells that produces IFN-γ & chemokines and dendritic cells activate T helper & cytotoxic T cells [75]. Some tumor variants, however, survive this surveillance and enter the equilibrium phase, where immune pressure maintains tumor cells in a dormant state mediated largely by IL-12, IFN-γ, and T cells [75, 76]. Over time, tumor clones with reduced immunogenicity adapt and progress to the escape phase, characterized by immune evasion mechanisms such as T cell exhaustion, PD-1/PD-L1 signaling, regulatory T cells (Tregs) are

recruited and M2 tumor-associated macrophages [77]. Hypoxia and HIF-1α signaling further promote immunosuppression by inducing IL-10, TGF-β, and immune checkpoint molecules [78]. Thus, immunoediting of the tumor results in an increased antitumor immunity while a non-edited tumor may evolve as immune ignorant (Fig. 4) [79]. In the antitumor immunity, the tumor can be considered as "immune-hot" and in the systemic immune ignorance, the tumor can be considered as "immune-cold" [80].

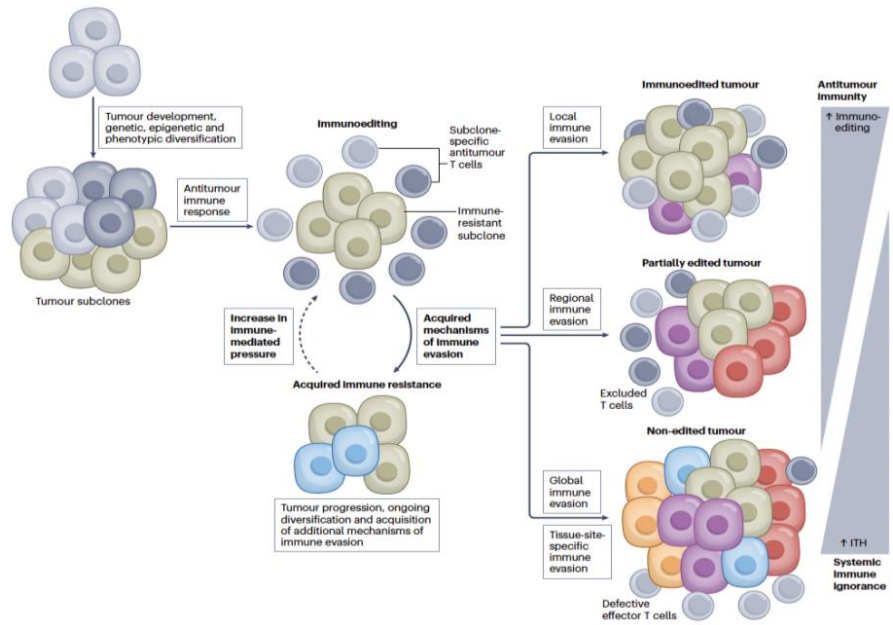

Figure 4: Immunoediting, tumor evasion and intratumor heterogeneity. Reprinted from Roerden et al. Cancer immune evasion, immunoediting and intratumour heterogeneity. Nature Reviews Immunology 2025 [79] with permission from Springer Nature.

In the context of leukoplakia progressing to OSCC, immunoediting is evident in shifts within the tumor microenvironment. In dysplastic and OSCC lesions there is an increased infiltration of CD8 positive- and CD4 positive T cells, but also B cells, reflecting active surveillance [81]. Previous studies from our group have also showed similar results, non-transforming leukoplakia had higher CD3 and CD8 expression [82]. However, when the immune landscape becomes increasingly immunosuppressive and IgA/IgG-secreting B cells decline [83], while

CD25+FoxP3+Tregs were correlated with leukoplakia progressing to OSCC [84]. Also, there is a rise in PD-1/PD-L1 and impaired cytotoxic response [85]. Tumor-associated macrophages particularly $CD163^{+}/CD204^{+}$ M2 macrophages accumulate and correlate with dysplasia severity and Treg presence reinforcing suppression [86]. Other immune cells such as mast cells, dendritic cells, myeloid-derived suppressor cells, eosinophils and neutrophils contribute variably, either enhancing immune surveillance or fostering tumor-promoting inflammation and angiogenesis [87]. Additionally, cancer-associated fibroblasts secrete TGF-β and other cytokines that shape an immunosuppressive microenvironment, driving immune tolerance and invasion [88].

Thus, during the transition from leukoplakia to OSCC, cancer immunoediting shifts from protective elimination to immune escape, with progressive remodeling of immune infiltrates, checkpoints, and stromal interactions that allow malignant transformation and tumor progression [87].

1.6 EPIGENETIC REGULATION IN CANCER: FOCUS ON EZH2

Epigenetics is defined as "the inheritance of DNA activity that does not depend on the naked DNA sequence" [89]. Epigenetics refers to heritable regulation of gene activity without altering DNA sequence, mediated by histone modifications, DNA methylation, non-coding RNAs and chromatin remodeling [90]. Cancers display global DNA hypomethylation at repetitive regions alongside promoter CpG island hypermethylation of tumor suppressor genes such as *BRCA1, hMLH1,* and *p16* [91]. Clinically, one of the earliest breakthroughs was the recognition that MGMT promoter hypermethylation predicts tumor sensitivity to chemotherapy [92]. Silencing also extends to growth-inhibitory microRNAs and other non-coding RNAs, weakening tumor surveillance [93, 94].

Advances in methylation arrays and whole-genome bisulfite sequencing permit detailed cancer methylome profiling [95]. Importantly, mutations in epigenetic regulators further drive malignancy, including DNMT3A [96], DNMT3B [97], EZH2 [98], UTX [99], ARID1A [100], and

PBRM1 [101], while global shifts in histone modifications reshape chromatin architecture [102]. Together, these epigenetic disruptions highlight how regulators such as EZH2 can function as oncogenic drivers, making the epigenome a critical target for cancer diagnostics and therapy.

1.6.1 EPIGENETICS IN ORAL SQUAMOUS CELL CARCINOMA

Epigenetic regulation including histone modifications and DNA methylation, controls chromatin structure and expression of gene without altering the DNA sequence [103]. In OSCC and its precursor lesions, this regulation is disrupted, with global hypomethylation activating oncogenes [104] and promoter hypermethylation silencing tumor suppressors such as *p15, p16, MGMT, hMLH1,* and *E-cadherin* [105, 106]. These alterations have also been detected in saliva and blood, making aberrant methylation a potential biomarker for early detection, prognosis, and disease monitoring [107, 108].

In addition to DNA methylation, abnormal histone modifications further reshape the OSCC epigenome. Overactive HDACs (e.g., HDAC2, HDAC6) and changes in acetylation or methylation marks (H3K4, H3K9, H3K27, H3K36) contribute to chromatin remodeling and tumor progression [103]. Dysregulation of key histone-modifying enzymes such as EZH2, which catalyzes H3K27 trimethylation, is correlated with poor prognosis in OSCC [109]. Since these modifications are reversible, epigenetic therapies including DNMT inhibitors, HDAC inhibitors, and EZH2-targeted drugs are being explored for OSCC management [110, 111].

Early detection of OSCC can be aided by epigenetic biomarkers, especially DNA methylation changes, detectable in saliva and tissues due to their non-invasive and predictive value [112, 113]. Tumor suppressor genes, including *p14, p15, p16, p73, DAPK, RASSF1A, MGMT, hMLH1, ATM, APC,* and *RUNX3* occurs through promoter region hypermethylation, affecting pathways like cell cycle, apoptosis, DNA repair, and Wnt signaling [114, 115]. Among these, *p16* methylation is a strong predictor of malignant transformation in precancerous lesions [116, 117]. Other markers include *FGFR1*

overexpression driven by hypomethylation [118, 119], *ATM, MSH6,* and *GATA5* promoter methylation linked to poor prognosis [120], and *RASSF1A* hypermethylation associated with oral cancer risk [121, 122]. Enhancer of zeste homolog 2 (EZH2) is overexpressed, and is consistently correlated with proliferation, metastasis, advanced stage, and poor survival in OSCC and other cancers [123, 124]. Genome-wide methylation studies further highlight panels of genes such as *Hox9, NID2, ZNF582, PAX1,* and a 12-gene signature (*SOX8, HORMAD2,* etc.) with potential diagnostic and prognostic value [125-127].

1.6.2 THERAPEUTIC TARGETING OF EPIGENETICS IN ORAL SQUAMOS CELL CARCINOMA

Epigenetic alterations are reversible and represent attractive therapeutic targets in OSCC. In head and neck squamous cell carcinoma, mutations in regulators like EZH2 drive aberrant histone methylation (H3K27me3) and tumor suppressor silencing [123, 124]. Drugs targeting these changes include DNA methyltransferase inhibitors (azacitidine, decitabine, guadecitabine) that reactivate silenced genes [128], histone deacytelase inhibitors (valproic acid, entinostat) that induce apoptosis [129, 130], and EZH2 inhibitors (tazemetostat) that block oncogenic histone methylation [131]. These epi-drugs are being explored as a standalone or combination therapies to improve OSCC management.

1.6.3 EZH2 IN LEUKOPLAKIA AND ORAL SQUAMOUS CELL CARCINOMA

EZH2 is a catalytic subunit and an important core component in polycomb repressive complex 2 (PRC 2), which is a group of polycomb proteins involved in gene silencing and regulates histone methylation of many genes [132]. EZH2 regulates downstream target genes by tri-methylation of histone 3 at lysine 27 (H3K27me3) [133]. EZH2 regulates the genes involved in the cell cycle progression, cellular differentiation, autophagy and apoptosis, thereby causing initiation of cancer, proliferation of cells, drug resistance, metastasis and also regulation of immune cells [134].

A study by Cao et al., reported that EZH2 expression predicts malignant transformation in patients with leukoplakia [135]. In a recent study Chen et al showed that HOXC9 contributes to the progression of leukoplakia into malignancy by enhancing cancer stem cells properties and promoting epithelial to mesenchymal transition, while HOXC-AS1 sustains this phenotype through EZH2-mediated H3K27me3 regulation, suggesting the HOXC-AS1/HOXC9/EZH2 axis as a potential therapeutic target [136].

In OSCC, EZH2 and BMI1 are significantly upregulated, with EZH2 expression linked to tumor invasion and p53 alteration. These findings suggest that p53 dysregulation may drive EZH2 overexpression, making it a potential early event in OSCC development [137]. In addition to EZH2, studies have explored the role of EZH1 in OSCC. EZH2 promotes proliferation, apoptosis, invasion, and metastasis [138], while EZH1 suppresses these traits. Their competition for PRC2 binding may explain prognostic differences, suggesting dual EZH1/2 inhibitors may be unsuitable for OSCC [139]. Another study on tongue cancer, also states that EZH2 is a key oncogenic driver, potential diagnostic and prognostic biomarker. DZNep exerts anticancer effects by degrading EZH2 and reversing PRC2-H3K27me3-mediated gene silencing, highlighting EZH2 as a promising therapeutic target [124].

1.7 TOLL-LIKE RECEPTORS IN ORAL EPITHELIUM AND DISEASE PROGRESSION

TLRs are a family of pattern recognition receptors that form a critical part of the innate immune system [140], expressed not only on immune cells but also on epithelial and tumor cells, where they modulate inflammation [141]. Toll-like receptors (TLRs) are transmembrane proteins composed of extracellular leucine-rich repeat regions responsible for ligand recognition and an intracellular Toll/IL-1 receptor (TIR) domain involved in signal transduction. [142]. In humans, ten types of TLRs have been described. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are primarily localized on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 reside within intracellular compartments [143].

TLRs are found not only on immune cells but also on epithelial and tumor cells, where they regulate inflammatory responses [141, 144].

Upon ligand binding, most TLRs activate the MyD88-dependent signaling pathway, while TLR3 and TLR4 can also signal through TRIF, ultimately leading to NF-κB activation and induction of immune-related genes (Fig. 5) [145]. Through these mechanisms, TLRs plays dual roles by initiating immune responses to tissue injury and pathogens, but they can also influence tumor biology, contributing to either progression or suppression [146, 147].

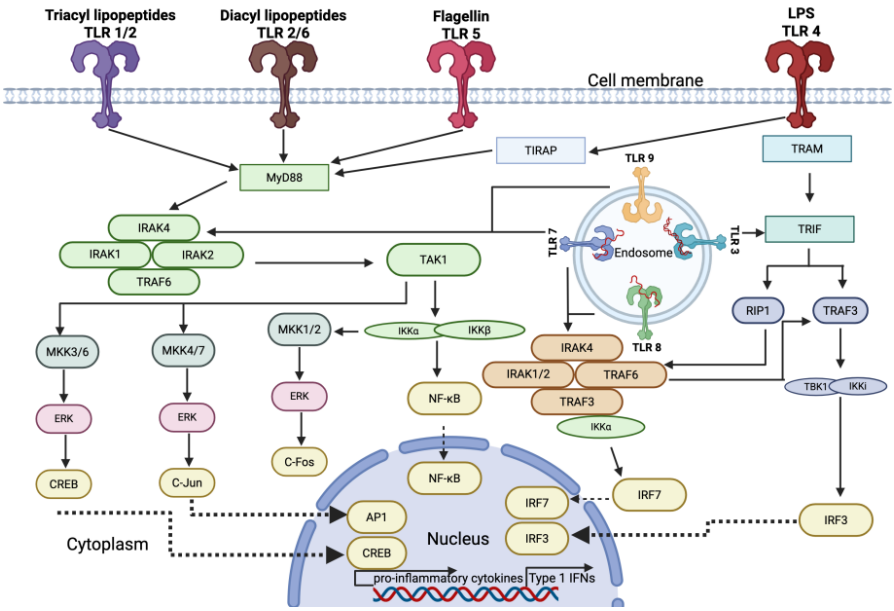

Figure 5: Toll-like receptor signaling. Adapted from Duan et al. Toll-Like Receptor Signaling and Its Role in Cell-Mediated Immunity [146]. Created in BioRender. Ganesh, D. (2025) https://BioRender.com/nodu34u

1.7.1 THERAPEUTICS TARGETS OF TOLL-LIKE RECEPTORS

Since 2018, at least 22 clinical trials in oncology testing TLR agonists in oncology have been initiated (source http://clinicaltrials.gov/). Agents targeting TLR3 (Ampligen, BO-112, Poly-ICLC) are under

investigation in ovarian, breast, melanoma, and advanced solid tumors, with most in Phase I or I/II trials**.** TLR7 and TLR7/8 agonists (NJH395, SHR2150, BDB001, BDB018) are being tested in multiple solid tumors, frequently in combination with checkpoint inhibitors, mainly in Phase I–II trials**.** TLR8 agonists (SBT6050, SBT6290) are under study in HER2+ cancers in Phase I and I/II trials. TLR9 agonists (SD-101, TAC-001, Tilsotolimod, Vidutolimod, CAS3/SS3) are being evaluated in lymphoma, melanoma, hepatocellular carcinoma, pancreatic, prostate, and other cancers, with ongoing Phase I–II studies [148].

Importantly, a Phase I trial of topical imiquimod (TLR7 agonist) is recruited for patients with oral cancer at early stage. Most of these trials are designed to assess safety, immunogenicity, and synergy with checkpoint inhibitors [148].

Numerous clinical studies have evaluated TLR agonists as adjuvants in cancer vaccines, demonstrating their safety and ability to enhance an immune response. The TLR3 agonist poly-ICLC has been widely tested, either alone or in combination with other agonists, in glioblastoma, melanoma, and breast cancer, where it activated dendritic cells and elicited robust antigen-specific CD4 positive and CD8 positive T-cell response (Phase I–II) [149-152]. TLR4 agonists such as GLA and MPLA have been incorporated into NY-ESO-1, KSA, and MART-1a vaccines, inducing strong antigen-specific antibody and T-cell responses in solid tumors (Phase I) [153-155]. TLR7 agonists, including imiquimod and resiquimod, have been tested in melanoma vaccines, showing induction of antigen-specific antibodies, CD8 positive T cells, NK cell activation, and even regression of metastasis in some Phase I–II trials [156-158]. The TLR9 agonist CpG7909 has been studied in melanoma and esophageal squamous cell carcinoma vaccines, with promising antigen-specific T-cell activation (Phase I–II) [159].

Collectively, these findings indicate that TLR agonists are promising cancer vaccine adjuvants, capable of boosting both humoral and cellular immunity across a range of tumor types.

1.7.2 TOLL-LIKE RECEPTORS IN LEUKOPLAKIA

Evidence regarding TLR expression in leukoplakia is limited. Kotrashetti et al. reported low TLR9 expression in oral epithelial dysplasia and moderate levels in OSCC [160], while Ali et al. confirmed TLR expression in leukoplakia, showing that TLRs are detectable in both normal mucosa and leukoplakia with or without dysplasia [161]. Other agents such as poly I:C (TLR3 agonist), which induces apoptosis via IFN-β and caspase activation in OSCC cell lines [162, 163], suggest potential preventive applications in leukoplakia, though direct evidence is lacking. Importantly, the TLR7 agonist imiquimod has shown promising clinical results, with topical treatment reducing leukoplakia lesion size by ≥50% in two-thirds of patients, highlighting its potential as a chemo preventive therapy [23, 164, 165].

1.7.3 TOLL-LIKE RECEPTORS IN ORAL SQUAMOUS CELL CARCINOMA

In OSCC, TLRs contribute to carcinogenesis at multiple levels which has been shown in cell line studies, studies on genetic polymorphisms, and tissue analyses. In oral cancer HB cell line , TLR9 was shown to drive proliferation via the AP-1/cyclin D1 pathway [166], while TLR3/4 formed a positive feedback loop with HIF-1, enhancing tumor progression [167]. TLR2 activation promoted growth, invasion, and cisplatin resistance through the miR-146a pathway [168], and TLR5 linked bacterial stimuli to inflammation-driven tumor growth and poor prognosis [169].

Polymorphism studies highlight the genetic role of TLRs in OSCC susceptibility. TRAF6 rs5030486 was significantly associated with OSCC risk [170], while TLR3 single nucleotide polymorphisms (SNPs) rs3775290 and rs3775291 were also linked to disease susceptibility and pathogenic protein alterations [171]. TLR9 rs187084 CC genotype associated with metastasis of the lymph node, advanced stage disease, and poor prognosis, although not as an independent predictor, and both TLR9 rs187084 and TLR7 rs3853839 variants were associated with heavy alcohol consumption [172]. Additionally, TLR9 (−1486T/C) and TLR4 (+896A/G) polymorphisms, particularly in the context of

HPV/EBV co-infection and tobacco exposure, further increased oral cancer risk [173].

In tissue studies, overexpression of TLR3 was observed in OSCC lesions and linked to aggressive disease [174, 175], although Rusanen et al. reported that the TLR3, TLR4, TLR7 and TLR8 intensity staining was significantly reduced in basement membrane zone and epithelial layer of OSCC demonstrated a decreased intensity of staining in TLR1, TLR2, TLR5, and TLR8 in comparison to the healthy control tissues [176]. However, Daskalopoulos et al, stat es that high expression of TLR4, and TLR9 was consistently found in tumor samples [177]. Furthermore, tumors with high TLR9 expression in heavy alcohol consumers displayed fewer CD8 positive T cells intratumorally despite similar overall infiltration, suggesting altered chemokine expression, impaired CD8 positive T cell activation, or alcohol-enhanced protumorgenic TLR9 signaling [178].

TLR agonists have also been tested in OSCC cell lines. TLR3 activation by poly I:C induces apoptosis and inhibits growth in OSCC through interferon-β and caspase activation [162, 163]. TLR7 activation by imiquimod significantly inhibited proliferation by inducing mitochondria-dependent apoptosis [165]. TLR8 activation by CL075 has not been investigated in OSCC, but in cervical cancer derived HeLa cells, 48-hour exposure to the agonist caused cells to cluster in G2/M and S phases, indicating enhanced proliferative activity [179]. TLR9 activation by CpG ODN 2006 (TLR9) can activate antitumor immunity but has also been implicated in immunosuppression and tumor progression via the PARP1/PD-L1 axis [180, 181].

Together, these studies demonstrate that TLRs function as a double-edged sword in OSCC acting as drivers of tumor progression and therapy resistance but also offering opportunities as biomarkers and targets for immunotherapy.

1.8 INTERPLAY BETWEEN EPIGENETIC MODULATION AND IMMUNE SIGNALING

EZH2 functions as a key mediator of immune cell activation and inflammatory responses. In macrophages, EZH2 sustains a TLR4–NF-κB circuit, enhancing cytokine production and host protection, whereas its loss in neutrophils impairs antibacterial defense, highlighting paradoxical roles across myeloid lineages [182]. Inhibition with EPZ-6438 significantly reduced inflammatory gene expression in microglia and macrophages stimulated with LPS, suggesting immunomodulatory potential [183]. Mechanistically, EZH2 suppresses *Socs3* via H3K27me3, and its loss induces *Socs3* upregulation, TRAF6 degradation, and reduced TLR–MyD88–NF-κB signaling, thereby dampening macrophage/microglial activation and suppress the autoimmune inflammation [184].

Few studies, have demonstrated that EZH2 inhibition induces intracellular dsRNA, leading to activation of innate immune signaling pathways involving TLR3 and STING pathways. Studies have shown that reduced EZH2 activity is associated with increased expression of TLR3 and other dsRNA sensors, thereby enhancing interferon signaling and promoting antitumor immune responses across multiple cancer types [185, 186].

In NK progenitor cells, EZH2 deletion upregulated 532 genes, including *Klrk1* (NKG2D), *IL2ra*, *IL7r*, multiple chemokine receptors (*Cxcr3, Ccr7*), TLRs (*Tlr3, Tlr8*), and cytotoxicity-related proteases (*Gzma, Gzmb*), enhancing NK cell development and effector function [187]. Similarly, in dendritic cells, EZH2 and EED regulate H3K27me3 at the *LRRC33* promoter, where EZH2 silencing increased *LRRC33* expression, while MLL/RBBP5 silencing reduced *LRRC33* expression through loss of H3K4me3 [188].

Taken together, these findings underscore that EZH2 modulates immune responses through epigenetic influence on TLR signaling and related pathways, linking its role in inflammation to tumor progression. Possibly, dysregulation of EZH2–TLR interactions in OSCC may

represent a critical mechanism driving inflammation-associated carcinogenesis and may be a potential target for therapy.

1.9 PROTEOMIC PROFILING

1.9.1 SALIVARY PROTEOMIC ANALYSIS

Saliva has been extensively studied as a non-invasive biofluid for biomarker discovery in leukoplakia and OSCC. Most proteins detected are abundant salivary components such as amylase, cystatins, albumins, and immunoglobulins; however, Cytokeratin 10 fragments emerged as novel leukoplakia markers [189]. In OSCC saliva, iTRAQ-MS identified 93 elevated proteins, and eight were selected, including CD44, S100A7, and S100P, were validated as potential early detection markers for dysplastic leukoplakia and OSCC [190]. A meta-analysis further highlighted carcinoembryonic antigen (CEA) and CYFRA21 as clinically useful, with CYFRA21 uniquely capable of distinguishing OPMDs from healthy controls [191]. More recently, 14-3-3 Tau-protein (YWHA), calreticulin (CALR), and ribosomal protein RACK1 were implicated in proliferative verrucous leukoplakia, with CALR and RACK1 possibly playing distinct roles in leukoplakia with and without dysplasia [192]. These results reinforce saliva as a practical possible diagnostic tool for monitoring disease progression and early OSCC detection.

1.9.2 PROTEOMIC INSIGHTS INTO LEUKOPLAKIA MALIGNANT TRANSFORMATION AND ORAL SQAMOUS CELL CARCINOMA

Proteomic studies have identified several candidate biomarkers for malignant transformation of leukoplakia into OSCC. Dong et al. quantified 34 differential proteins (18 upregulated, 16 downregulated), including 13 reported for the first time, implicating pathways such as Rho GTPase signaling, mitochondrial dysfunction, and 14-3-3-mediated signaling in leukoplakia progression [193]. In a preliminary study from 2023, dysregulated proteins included LDHA, which drives glycolysis to support proliferation; TXN, which disrupts redox balance leading to

genomic instability; PLEC, upregulated to promote proliferation and invasion; SPTAN1, downregulated to weaken cell–cell adhesion; and WDR1, which enhances actin remodeling and motility. These findings suggest that cytoskeletal regulation, oxidative stress, and extracellular matrix remodeling are central to leukoplakia progression [194]. A consecutive study by Sharma et al., states that, 2,685 groups of protein and 12,397 peptides were identified, with 61 proteins showing consistent expression across control, leukoplakia, and OSCC phenotypes. Three proteins displayed linear changes across phenotypes: Vimentin (VIM), Collagen type VI alpha 2 chain (COL6A2) and Fibrinogen β chain (FGB) increased from leukoplakia to OSCC [195].

1.10 NUCLEAR-CYTOPLASMIC SHUTTLING OF PROTEIN BIOMARKERS AND THEIR FUNCTIONAL ROLES

In eukaryotic cells, the nuclear pore complex (NPC) controls the transport between the nucleus and cytoplasm, allowing replication and transcription to occur within the nucleus while translation takes place in the cytoplasm. [196]. Since then, many transcription factors, proliferation regulators, hormone receptors, and RNA-binding proteins have been shown to shuttle across the nuclear envelope [197, 198]. Shuttling proteins primarily serve two key functions. First, they facilitate the transport of specific RNAs and proteins across the nuclear membrane [199]. Second, they coordinate the transfer of regulatory information between the nucleus and cytoplasm, thereby linking nuclear events with cytoplasmic functions [200].

NPCs, composed of ~30 nucleoporins, form large protein channels that regulate traffic across the nuclear envelope [201]. They permit passive diffusion of small molecules (<40 kDa) but require active, energy-dependent transport for larger cargos such as proteins and RNAs [202]. The active transport of proteins between the nucleus and cytoplasm is regulated by specific signal sequences nuclear localization signals that facilitate import into the nucleus and nuclear export signals that promote their release to the cytoplasm. These signals are recognized by nuclear transport receptors, also known as members of the karyopherin/importin exportin family, which act as transport mediators [203].

Transport directionality depends on the Ran GTPase system, which maintains a RanGTP gradient (high in the nucleus, low in the cytoplasm). RanGTP binding or hydrolysis determines whether a transport complex assembles or disassembles, ensuring cargos are released in the correct compartment [204].

For nuclear import, the classical pathway involves the importin α/β heterodimer: importin-α binds to the nuclear localization signals on the cargo protein, while importin-β interacts with nucleoporins in the NPC, shuttling the complex into the nucleus. Once inside, RanGTP binds importin-β, causing cargo release. For nuclear export, the receptor CRM1 (exportin 1) recognizes leucine-rich nuclear export signals-containing proteins, forming a complex with RanGTP and the cargo, which is transported out of the nucleus. Hydrolysis of RanGTP in the cytoplasm then triggers cargo release [203].

Beyond these, specialized exportins regulate specific cargos: exportin-t exports tRNAs, exportin-5 mediates pre-miRNA export, and others handle transcription factors and adaptor proteins [203]. Collectively, nucleocytoplasmic transport signifies a central regulatory system that coordinates nuclear and cytoplasmic activities ensuring accurate gene expression, protein localization, and signaling. Its dysregulation contributes to cancer, viral infection, and other diseases, highlighting its biomedical importance [205, 206].

Multiple studies highlight the role of altered protein expression and localization in oral carcinogenesis. Nuclear SNAIL expression increases from normal oral mucosa to leukoplakia, but decreases with a shift to cytoplasmic expression in OSCC, while cytoplasmic E-cadherin is significantly elevated in OSCC and lymph nodes, marking malignant transformation [207]. Survivin expression is higher in both cytoplasm and nucleus of OSCC compared to normal oral mucosa and leukoplakia, suggesting early anti-apoptotic activity, while nuclear survivin and caspase-3 overexpression in OSCC indicate enhanced proliferation and apoptosis at later stages [208]. Abnormal PER2 localization and phase shifts, even in adjacent noncancerous tissues, support its tumor-suppressive role via TP53 activation and inhibition of epithelial to mesenchymal transition [209]. CLLD7 and CHC1L showed altered nuclear-to-cytoplasmic or membrane localization in OSCC [210].

Autophagy-related proteins also appear distinct: LC3A was more frequent in non-dysplastic lesions, whereas LC3B was associated with dysplasia and significantly elevated in OSCC, suggesting divergent roles in progression [211].

1.11 INTEGRATING MULTI-OMICS APPROACHES IN ORAL CANCER RESEARCH

Multi-omics strategies including transcriptomics, proteomics, metabolomics and genomics have advanced our understanding of OSCC biology and biomarker discovery.

Advances in **genomics** have revealed frequent alterations in oral cancer, including TP53 mutations, EGFR changes, CDKN2A deletions, chromosomal abnormalities, microsatellite instability, and promoter hypermethylation of tumor suppressor genes [163, 212]. These changes are detectable in saliva as well as tissue, supporting the development of non-invasive biomarkers.

Transcriptomics has highlighted dysregulated expression of coding and non-coding RNAs, with upregulation of oncogenes (e.g., MYC, IL8, SAT, miR-31) [213-215] and downregulation of tumor suppressors (e.g., CDKN2A, miR-375) [216]. These signatures not only reflect tumor progression but also predict therapy resistance and prognosis.

In **proteomics**, deregulated proteins involved in glycolysis, cytoskeletal regulation, stress responses, and inflammation have been identified in tissue, serum, and saliva. Markers such as CD44, S100A7, S100P, and survivin show strong potential as early detection and prognostic indicators, particularly through salivary proteomics [214, 217].

Metabolomics studies, though fewer, consistently point to altered energy and amino acid metabolism in OSCC. Increased glycolysis- the Warburg effect, disrupted lipid pathways, and metabolites like valine, lactate, and phenylalanine correlate with malignant transformation and may provide diagnostic and therapeutic leads [218].

Taken together, integrative omics approaches offer a comprehensive view of oral carcinogenesis. Combining genomic data (e.g., TP53 mutations) with transcriptomic, proteomic, and metabolomic profiles enables identification of biomarkers, therapeutic targets, and predictors of treatment response. While promising for personalized and precision oncology, these findings require validation in larger cohorts and cross-omics integration to enable routine clinical translation.

1.12 CURRENT GAPS IN DIAGNOSIS AND TREATMENT OF LEUKOPLAKIA

1.12.1 CURRENT GAPS IN DIAGNOSIS OF LEUKOPLAKIA

1. **Lack of reliable biomarkers**
    - No universally validated molecular or proteomic biomarkers exist to predict malignant transformation risk with high accuracy.
    - Current histopathology (grading of dysplasia) is subjective and prone to inter-observer variability.
2. **Heterogeneous disease nature**
    - Leukoplakia lesions vary in clinical appearance, histology, and molecular features, making risk stratification challenging.
    - Not all leukoplakia with dysplasia progress to OSCC, while some non-dysplastic leukoplakia does transform.
3. **Non-invasive tools underdeveloped**
    - Salivary and liquid biopsy biomarkers (DNA methylation, miRNAs, proteins) show promise but lack clinical validation and standardized protocols.
    - Imaging-based diagnostic tools (e.g., autofluorescence, optical coherence tomography) are not yet accurate or widely used in clinical practice.

1.12.2 CURRENT GAPS IN THE TREATMENT OF LEUKOPLAKIA

1. **No standard therapeutic guidelines**
    - Management varies widely (surgical excision, laser ablation, observation, chemoprevention) due to lack of consensus on best practices.
2. **High recurrence and progression rates**
    - Even after surgical removal, recurrence is common, and some patients still progress to OSCC.
3. **Limited role of chemoprevention**
    - Agents like retinoids, COX-2 inhibitors, and green tea polyphenols have been tested, but none are established in routine care due to modest efficacy and side effects.
4. **Poor risk stratification for treatment decisions**
    - Patients with low-risk lesions may undergo unnecessary invasive procedures, while high-risk cases may be missed due to absence of precise molecular predictors.
5. **Lack of integration of multi-omics**
    - Genomics, transcriptomics, proteomics, and metabolomics offer insights, but their clinical translation into personalized therapy is still limited.

1.13 RATIONALE AND SIGNIFICANCE OF THE STUDY

Current diagnostic tools primarily rely on histopathological grading of dysplasia, which suffers from subjectivity and limited predictive value, as even non-dysplastic lesions can transform. This highlights the urgent need for reliable molecular and cellular biomarkers to improve risk stratification and guide patient management.

This thesis addresses this gap by investigating multiple layers of tumor biology in leukoplakia and OSCC. **Paper I** explores the role of the epigenetic regulator EZH2, assessing its association with malignant transformation and its correlation with T cell infiltration, thus linking epigenetic dysregulation with immune responses in leukoplakia. **Paper**

II examines intracellular TLRs and immune cells in leukoplakia and OSCC, comparing expression and localization in leukoplakia with and without dysplasia and OSCC. Thus, providing insights into innate immunity in premalignant and malignant disorders. **Paper III** extends this work to oral epithelial cell lines, determining how TLR expression and stimulation influence cellular proliferation, thereby identifying potential mechanistic pathways underlying tumor progression. **Paper IV** applies a proteomic approach, comparing the protein expression profiles of transforming versus non-transforming leukoplakia patients over long-term follow-up, with the aim of identifying novel biomarkers predictive of malignant transformation.

Collectively, these studies integrate epigenetic, immunological, and proteomic perspectives to provide a comprehensive understanding of leukoplakia progression into OSCC.

2 AIM

The overall aim of this thesis was to investigate biomarkers associated with oral leukoplakia and evaluate their role in malignant transformation.

2.1 SCIENTIFIC QUESTIONS

Paper I

-Is there an association between EZH2 expression and cancer transformation in leukoplakia?

-Is there a correlation between EZH2 expression and T-cell infiltration in leukoplakia?

Paper II

-Does the presence and frequency of intracellular Toll-like receptors and different subsets of T cells influx in epithelium differ between leukoplakia with, without dysplasia and OSCC?

-Is there a nuclear and/or cytoplasmic expression of intracellular Toll-like receptors that differ between leukoplakia with and without dysplasia?

Paper III

-Does the expression of Toll-like receptors differ between malignant and pre-malignant oral epithelial cell lines?

- Does stimulation of intracellular Toll-like receptors alter proliferation in malignant and pre-malignant oral cell lines?

Paper IV

-Does the proteomic profile differ between leukoplakia patients who transform into OSCC and those who remain non-transforming during five years of follow-up?

- Are specific top-regulated proteins identifiable in leukoplakia transforming into OSCC and leukoplakia not transforming to OSCC?

3 PATIENTS AND METHODS

3.1 PATIENTS

Papers I and II

Patients with clinical diagnosis of leukoplakia, histopathological diagnosis of hyperkeratosis (with or without dysplasia) (n=23) were collected. During follow-up period of at least six months, nine patients progressed to OSCC (OL-ca), while nine remained OSCC free (OL-non) for at least five years. A re-evaluation of histopathology led to the exclusion of three patients due to revised diagnoses, insufficient or damaged samples in two patient, final analysis included (n=18) in paper I.

Patients with clinical diagnosis of leukoplakia (n=34) were collected, based on histopathological diagnosis patients were divided into hyperkeratosis with dysplasia (OL-dys) (n=13), benign hyperkeratosis (OL-no) (n=21) and histopathological diagnosis of OSCC (n=11) were collected. After a blind re-evaluation, three OL-dys cases were reclassified as OL-no, and one OSCC case was excluded in paper II. Dysplasia severity was assessed by a senior pathologist. Tissue specimens were retrieved retrospectively from the archives of the department of Oral Medicine and Pathology, Institute of Odontology, University of Gothenburg and Department of Pathology, Sahlgrenska University Hospital, Sweden. One OSCC patient had signs of fungal infections by PAS staining.

Paper IV

A longitudinal multicenter (ORA-LEU-CAN) prospective study, patients with clinical diagnosis of leukoplakia, histopathological diagnosis of hyperkeratosis (with or without dysplasia) (n=22) were collected. During follow-up period of at least five months, ten patients progressed to OSCC (leuko-ca), while twelve remained OSCC free for at least five years (leuko-nonca).
In all three papers, baseline leukoplakia biopsies and tumor biopsies were analyzed from paraffin-embedded samples stored at Department of Pathology, Sahlgrenska University Hospital, Sweden.

3.2 ETHICAL CONSIDERATIONS

The Swedish Ethical Review Authority, Sweden approved the study Dnr. 2019-04579, 2020-04405 (Study I, II). In their approval, the Swedish Ethical Review Authority waived informed consent from the patients since this is a retrospective study on historical patients over a long time period. The Regional Ethics Review Board in Gothenburg, Sweden approved the study Dnr.673–10, T864-11(Study IV) and informed consent from the patients were obtained since this is a prospective study. These studies were conducted in accordance with the Declaration of Helsinki.

3.3 CELL LINES

Paper III

Two malignant cell lines SCC25 (Squamous cell carcinoma 25), CalH3 (Cellosaurus cell line) and one premalignant cell line DOK (Dysplastic Oral Keratinocytes) were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 10 ng/ml epidermal growth factor, 0.4 µg/ml hydrocortisone, 5 µg/ml insulin-ferritin-transferrin, 50 µg/ml ascorbic acid, and 2% L-glutamine.

3.4 METHODS

3.4.1 **CELLULAR IMAGING**

Paper I

Immunofluorescence was performed on 4µm thick formalin-fixed and paraffin embedded tissue samples. The tissues were stained with EZH2 mouse monoclonal antibodies (Cat. No. 612666; BD Transduction Laboratories, Stockholm, Sweden). Immunohistochemistry was also performed with rabbit monoclonal antibodies targeting the immunological markers CD3, CD8 (MA5-14524, MA5-14548; Thermo Fisher Scientific, Gothenburg, Sweden) and CD1a (Cell Marque, EP3622; Sigma Chemical Co., Stockholm, Sweden). Goat serum was added to the tissue instead of primary antibody acted as a negative control.

Paper II and IV

Immunohistochemistry was performed on 4μm thick formalin-fixed and paraffin embedded tissue sections. The tissues were stained with TLR3 rabbit monoclonal antibody (ab62566; Abcam, Cambridge, UK), rabbit polyclonal antibodies TLR7, TLR8 (PA5-95046, PA5-102413; Thermo Fisher, Gothenburg, Sweden) and TLR9 mouse monoclonal antibody (ab134368; Abcam, Cambridge, UK). Rabbit monoclonal antibodies targeting the immunological markers CD3, CD8 MA5-14524, MA5-14548; Thermo Fisher Scientific, Gothenburg, Sweden) were also used in immunohistochemical staining. Healthy control samples were used as positive control for CD3, CD8 and goat serum was added to the tissue samples instead of primary antibody acted as a negative control (Paper II).

The tissue was stained with Kininogen 1 (PA5-81961; Thermo Fisher, Gothenburg, Sweden) rabbit polyclonal antibody and Apolipoprotein E (ab1906; Abcam, Cambridge, UK), Perlecan (13-4400; Thermo Fisher, Gothenburg, Sweden), EEFID (MA5-27512; Thermo Fisher, Gothenburg, Sweden) mouse monoclonal antibodies. The serum was added to the tissue samples instead of primary antibody acted as a negative control (Paper IV)

Paper III

Immunocytochemistry was performed on SCC25, CalH3 and DOK cell lines. These cell lines were stained with TLR3 rabbit monoclonal antibody (ab62566; Abcam, Cambridge, UK), rabbit polyclonal antibodies TLR7, TLR8 (PA5-95046, PA5-102413; Thermo Fisher, Gothenburg, Sweden) and TLR9 mouse monoclonal antibody (WH0054106M3; Sigma-Aldrich, Stockholm, Sweden) mouse monoclonal antibody. Goat serum was added to the cells instead of primary antibody acted as a negative control.

3.4.2 CELL QUANTIFICATION

Paper I

Digital images from blinded slides, three regions (one central and two peripheral) in the epithelial tissue, and a region (one central) in the

connective tissue were imaged at 200× magnification using an immunofluorescence microscope (Eclipse 90i; Nikon Inc., Tokyo, Japan). Image analysis was carried out using QuPath software, EZH2, CD3, CD8, CD1a positive cells were manually counted and the median numbers of positive cells/mm^2 were reported as a results in both the epithelium and connective tissue.

Paper II

Digital images of leukoplakia tissue were obtained, in from two to three high-power fields (HPFs) (one central and /or two peripheral) and in OSCC tissue three randomly selected tumor islands were imaged at 200× magnification using a light microscope (Leitz Wetzlar, Leica Microsystems) with a digital camera (UC30; Olympus Microsystem). Semi-quantitative assessment based on percentage was used to assess TLRs (TLR 3, 7, 8 and 9) in both leukoplakia and OSCC was scored as 0 (absent), 1 (≤33% positive cells), 2 (>33% to <67%), or 3 (≥67%) with mean scores recorded per patient. The nuclear and/or cytoplasmic expression scores staining of TLRs (TLR 3, 7, 8 and 9) were assessed and recorded as Neg: negative, Nuc: positive nuclei, Cyt: positive cytoplasm and Nuc & Cyt: positive nuclei and cytoplasm. In OL-no and OL-dys the highest score for each patient was registered. CD3, CD8 positive cells were manually counted and the numbers of positive cells/mm^2 were reported as a results in the epithelium.

Paper III

Digital images of SCC25, CalH3 and DOK cells were imaged at 200× magnification using an immunofluorescence microscope (Eclipse 90i; Nikon Inc., Tokyo, Japan), three random regions were chosen. Semi-quantitative assessment based on the expression was used to assess expression of TLR3, 7, 8, 9; 0- no expression, 1- low expression, 2- moderate expression and 3- high expression.

Paper IV

Digital images of leukoplakia tissue were obtained, a qualitative assessment of protein expression was conducted, which included the area of positive staining in either epithelium or connective tissue.

3.4.3 MOLECULAR TECHNIQUES

Paper III

The proliferation assay was performed using 100 µl cell suspensions from three cell lines, seeded at 40,000 cells per well in a 96-well plate. TLR agonists at three different concentrations (10 µl) were added and incubated for 24 hours. The next day, MTT reagent was added, followed by the addition of a cell proliferation reagent after 4 hours. Absorbance was measured using a Thermo Fisher Multiskan GO.

RT-qPCR assay was performed using RNA extracted from three cell lines (after incubating the cells with different agonists). A TaqMan real-time PCR assay targeting TLR3, TLR7, TLR8, TLR9, MyD88, TICAM1 was used to identify the intracellular TLRs and their downstream regulators, in addition five control genes were used, i.e., GADPH, GUSB, ACTB, MRPL19 and PUM1. Positive detection of target genes was defined as a threshold Ct value of <40.

Paper IV

Tissue sections (ten to fifteen sections) of 4 µm were deparaffinized, and proteins were extracted using TEAB-SDS lysis buffer. Protein concentration was measured via BCA assay, followed by digestion using a modified FASP method with trypsin. Peptides were labeled with TMTpro 18-plex reagents, purified, and fractionated using reversed-phase chromatography. Nano-LC-MS analysis was performed on an Orbitrap Fusion™ Tribrid™ mass spectrometer, with peptides separated on a C18 column. Precursor ions were fragmented by CID and HCD for MS3 analysis, and data were processed for protein identification. Proteome Discoverer 2.4 identified and quantified proteins with a 1% FDR threshold. Leukoplakia cases were classified into Leuko-nonca, LD-Leuko-ca, and SD-Leuko-ca.

3.4.4 STATISTICAL ANALYSIS

Statistical analysis was conducted using GraphPad Prism v9.2.0 (GraphPad Inc., San Diego, CA, USA). A $P<0.05$ was considered statistically significant.

Paper I

Spearman correlation was used to analyze the relationship between EZH2 expression and positive immune cell, with correlation graphs plotted after log10 transformation. The difference between two groups were assessed using Mann-Whitney U-test. Prognostic value of EZH2 expression was evaluated through receiver operating characteristic (ROC) curve analysis. Cancer-free survival was determined using Kaplan-Meier analysis.

Paper II

The Kruskal-Walli's test was used to compare antigen expressions between OL-no, OL-dys and OSCC. The Fisher's exact test was used to analyze nuclear and/or cytoplasmic expression in OL-no and OL-dys. The Mann-Whitney U-test was used to compare CD3, CD8 -expressing cells between the OL-no and OL-dys group.

Paper III

The Kruskal-Walli's test was used in RT-qPCR to analyze different agonist stimulation in cells and control in target genes.

Paper IV

STRING analysis and volcano plots identified key pathways and proteins. Proteomics values were log-transformed (base 2), and differences between groups were assessed using the student's paired t-test.

4 RESULTS

4.1.1 STUDY I

Is there an association between EZH2 expression and cancer transformation in leukoplakia?

Epithelial and stromal EZH2 expression levels were significantly elevated in leukoplakia cases that later transformed into OSCC compared to that leukoplakia that did not transform. High EZH2 expression showed strong predictive value for malignant transformation (AUC = 0.97, p = 0.0007) with 89% specificity and sensitivity. Moreover, patients with high EZH2 expression had significantly shorter cancer-free survival (p = 0.001) (Fig. 6) and a markedly 12.8-fold increased risk of OSCC development (HR = 12.8; 95% CI: 3.3–49.1)

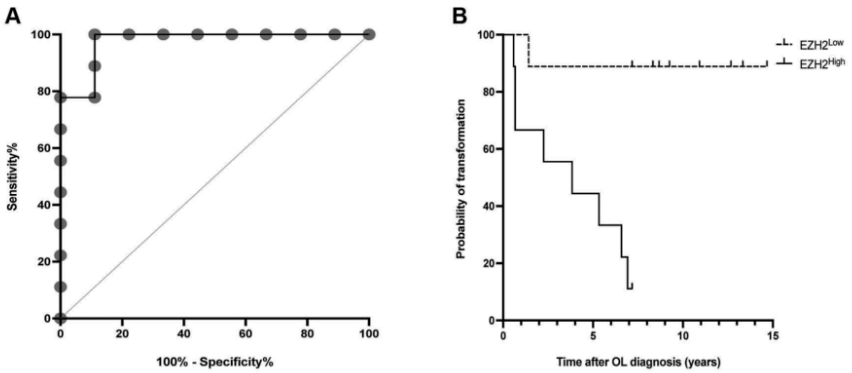

Figure 6: (A) Receiver operating characteristic curve illustrating the diagnostic accuracy of epithelial EZH2 expression. (B) Kaplan–Meier plot depicting malignant transformation following OL diagnosis, with censored cases indicated by ⊥ [219].

Is there a correlation between EZH2 expression and T cell infiltration in leukoplakia?

In the epithelium, EZH2 expression showed a moderate positive correlation with both CD3 positive T cells (r = 0.57; p = 0.01) and CD8 positive T cells (r = 0.59; p = 0.01), whereas no significant correlation was observed between EZH2 and CD1a molecule expression in the epithelium. In the connective tissue, CD3 positive and CD8 positive cells formed subepithelial lymphoid infiltrates, while CD1a positive

cells were infrequent. However, none of the immune markers CD3, CD8, or CD1a demonstrated a significant correlation with EZH2 expression in the connective tissue.

4.1.2 STUDY II

Does the presence and frequency of intracellular TLRs and different subsets of T cells influx in epithelium differ between leukoplakia with, without dysplasia and OSCC?

Expression levels of TLR3, TLR7, TLR8, and TLR9 did not differ significantly between leukoplakia without dysplasia, with dysplasia and OSCC. These findings indicate no changes in TLR expression across disease stages.

Is there a nuclear and/or cytoplasmic expression of intracellular Toll-like receptors differ between leukoplakia with and without dysplasia?

TLR3 showed similar nuclear and cytoplasmic expression in both OL-no and OL-dys ($p = 0.65$). In contrast, nuclear TLR7 expression was absent in OL-no but present in 31% of OL-dys cases ($p = 0.03$). Cytoplasmic TLR8 and TLR9 expressions were more frequently observed in OL-no (32% and 42%, respectively) than in OL-dys (8% and 23%, respectively), with statistically significant differences (TLR8: $p = 0.02$; TLR9: $p = 0.01$)

4.1.3 STUDY III

Does the expression of Toll-like receptors differ between malignant and pre-malignant oral epithelial cell lines?

All three oral epithelial cell lines DOK, SCC25, and CaLH3 expressed TLR3, TLR7, TLR8, and TLR9 at the protein level, with varying expression intensities. DOK cells showed moderate expression of TLR3, TLR7, TLR8, and TLR9. SCC25 cells had high TLR7 expression and moderate levels of TLR3, TLR8, and TLR9. CaLH3 cells had high TLR3 expression and showed moderate levels of TLR7, TLR8, and TLR9.

Gene expression analysis showed the expression of TLR3 and TLR9 in DOK and CaLH3, but only the expression of TLR9 in SCC25. Stimulation with TLR agonists downregulated TRIF in DOK ($p < 0.05$–0.01) and showed a similar trend in CaLH3 ($p = 0.09$), while MyD88 remained unchanged. TLR3 and TLR9 expression in DOK was reduced by CPG ODN2006 ($p < 0.05$), and TLR9 was further downregulated by poly I:C and imiquimod in both DOK and CaLH3 ($p < 0.01$–0.07).

Does stimulation of intracellular Toll-like receptors alter proliferation in malignant and pre-malignant oral cell lines?

Imiquimod showed the pronounced anti-proliferative effect, in a concentration-dependent manner reducing DOK cell growth and mildly affecting CaLH3 at high concentrations. In contrast, Poly I:C induced only a slight reduction of proliferation in the three assessed cell lines, and CL075 and CPG ODN2006 had no notable impact in any cell line.

4.1.4 STUDY IV

Does the proteomic profile differ between leukoplakia patients who transform into OSCC and those who remain non-transforming during five years of follow-up?

Several protein biomarkers showed significant differential expressions between leukoplakia transforming to OSCC within 5–26 months from diagnosis (short duration (SD)-leuko-ca) and non-transforming leukoplakia (leuko-nonca). Significantly lower expressions of Sortilin, collagen alpha-1(VII) chain, apolipoprotein E (ApoE), basement membrane-specific heparan sulfate proteoglycan (Perlecan) and apolipoprotein CII were observed in SD-leuko-ca compared to leuko-nonca. Conversely, eukaryotic elongation factor1-delta (EEF1D), lanC-like protein 2, mitochondrial Phosphoglycerate mutase 5 (PGAM5), ATP-binding cassette sub-family F member 2 (ABCF2) and serine/threonine-protein phosphatase PGAM5 were significantly more expressed in SD-leuko-ca compared to leuko-nonca. Kininogen-1 also showed a downward trend in SD-leuko-ca cases ($p = 0.097$).

In oral squamous cell carcinoma relative to non-transforming leukoplakia, proteins involved in antigen processing and presentation

were upregulated, whereas those associated with skin development and peptide cross-linking showed downregulation.

Are specific top-regulated proteins identifiable in leukoplakia transforming into OSCC and leukoplakia not transforming to OSCC?

Kininogen-1 showed cytoplasmic staining in the epithelium across all groups, with occasional staining in connective tissue cells. ApoE expression was weak, mainly in the connective tissue, epithelium and very weak cytoplasmic staining in OSCC. Perlecan was expressed in the cytoplasm of leuko-nonca, leuko-ca, and OSCC, particularly in tumor islands. EEF1D was observed to lesser extent in leuko-nonca, LD-leuko-ca and very weak cytoplasmic staining in SD-leuko-ca but showed strong nuclear staining in OSCC.

5 DISCUSSION

Paper I

A main finding in this study is the expression of the epigenetic regulator EZH2 in leukoplakia may function as a predictive biomarker for malignant transformation into OSCC. The study demonstrated that EZH2-expressing cells were significantly more frequent in leukoplakia cases that progressed to OSCC compared with non-transforming leukoplakia. Importantly, EZH2 expression was able to discriminate between non-transforming and transforming leukoplakia with a predictive accuracy of 97%, highlighting its potential value in risk assessment.

In addition, we observed a moderate positive correlation between EZH2 expressing cells and the presence of T lymphocytes expressing CD3 and CD8 markers were detected within the epithelial layer of tissues from patients with leukoplakia. This suggests that EZH2 may influence the local immune responses even at a premalignant stage. The CD8 molecule is primarily a marker for cytotoxic T cells, but it is also expressed to a lesser extent on NK cells and dendritic cells [220-222]. Since all these cell types are implicated in immune surveillance and in the elimination of dysplastic or transformed cells, their association with EZH2 highlights a potential mechanistic link between epigenetic dysregulation and immune activation. In established cancers, an association between EZH2-positive tumor cells and CD8 positive T cell infiltration has already been described, and it has been linked with patient prognosis [223]. The data in this study extend this observation to leukoplakia, pointing to a role for EZH2 in shaping the immune microenvironment at an earlier stage of oral carcinogenesis.

Importantly, within the tumor microenvironment, there exists an intricate interplay not only between tumor and immune cells but also involving other cell types, including fibroblasts and endothelial cells [224]. Thus, the epigenetic regulation of these cells present in the leukoplakia microenvironment may play a crucial role in immunosurveillance to prevent malignant transformation and warrants further investigation.

The comparison between transforming and non-transforming leukoplakia further confirmed the role of EZH2. In both the epithelial and connective tissue compartments, EZH2 expression was markedly higher in leukoplakia that developed into OSCC. This observation was observed by Cao et al., who reported a similar association in a Chinese patient cohort [135]. Moreover, our Kaplan–Meier survival analysis demonstrated that patients with $EZH2^{high}$ leukoplakia lesions had a significantly lower cancer-free survival than those with $EZH2^{low}$ leukoplakia. Importantly, patients with elevated EZH2 expression exhibited a 13-fold higher risk of developing OSCC, highlighting the significant prognostic potential of EZH2 expression.

From a clinical standpoint, these findings suggest that EZH2 may serve as a valuable biomarker for risk stratification in leukoplakia patients. Identifying individuals with high EZH2 expression could enable clinicians to recognize patients at greatest risk of malignant transformation and to monitor them more closely or initiate preventive interventions.

A main limitation is the small sample size. But also, the retrospective design which curb retrieval of complete clinical data, and the well-known subjectivity of dysplasia assessment in leukoplakia [225] are factors that curb the readouts from the study. The variability in grading of dysplasia highlights the need for objective biomarkers like EZH2 to support evaluation of histopathology.

Paper II

This study demonstrates that TLR3, TLR7, TLR8, and TLR9 are expressed in the oral epithelium of patients with leukoplakia, both with and without dysplasia, as well as in OSCC. Importantly, we observed distinct differences in subcellular localization depending on the lesion type. Nuclear expression of TLR7 was elevated in leukoplakia with dysplasia compared to leukoplakia without dysplasia, whereas cytoplasmic expression of TLR8 and TLR9 was reduced in leukoplakia with dysplasia. These findings suggest that changes in TLR localization may be associated with malignant progression.

Few investigations have addressed the significance of nuclear versus cytoplasmic localization of TLRs in oral carcinogenesis. Kauppila et al. reported that TLR5 expression changed from a localized to a more diffuse pattern during progression from normal epithelium to dysplasia and oropharyngeal squamous cell carcinoma [226]. In another study, cytoplasmic TLR5 expression was significantly higher in oral cancer compared with healthy oral epithelium [227]. Similarly, previous reports have shown TLR3 and TLR9 localized both in the cytoplasm and at the plasma membrane, while TLR7 was identified at the nuclear membrane or within the nuclei of oropharyngeal squamous cell carcinoma cases [228]. Consistent with these studies, data from this study indicate that in dysplastic leukoplakia, TLR3 and TLR7 are mainly nuclear or dual-localized, whereas in non-dysplastic lesions, TLR8 and TLR9 are largely cytoplasmic or dual-localized, with limited nuclear presence.

The precise cellular localization of TLRs is likely to be functionally relevant, as nucleocytoplasmic shuttling regulates key processes such as proliferation and apoptosis [203]. Proteins are transported between the cytoplasm and nucleus by nuclear import and export signals, and their localization often determines activity [203]. Based on our findings, we hypothesize that TLR7 may undergo active shuttling from cytoplasm to nucleus in leukoplakia with dysplasia, where it could trigger downstream pathways promoting dysplastic cell proliferation. In contrast, in leukoplakia without dysplasia TLR7 may remain in an inactive state, localized in the cytoplasm or partly in both compartments. Conversely, shuttling of TLR9 was less evident in leukoplakia with dysplasia, where it was mainly absent or confined to cytoplasmic and dual localization. In contrast, leukoplakia without dysplasia showed cytoplasmic or dual localization, suggesting a lack of nuclear translocation.

The role of TLRs in leukoplakia must also be considered in the broader context of immune activation. TLRs are central components of the innate immune system and influence adaptive responses, particularly T-cell recruitment [229]. In this study, we observed a trend toward higher numbers of CD3 positive T cells in leukoplakia with dysplasia compared to leukoplakia without dysplasia, although CD8 positive T cells did not differ significantly between groups. Öhman et al. previously reported

increased infiltration of both CD3- and CD8-positive T cells in leukoplakia with dysplasia [230]. Though the findings did not reach statistical significance, the observed trend suggests a role of TLR-mediated signaling in shaping the immune microenvironment of leukoplakia. This shaping may result in either an “immune-hot” or an “immune-cold” leukoplakia in analogy what has been suggested for established tumors [80].

Clinically, TLRs are emerging therapeutic targets, with agonists such as the TLR7 activator imiquimod already showing efficacy in oral potentially malignant disorders [23, 148, 231]. Our findings support the potential of targeting intracellular TLRs in high-risk leukoplakia as a preventive or therapeutic approach.

The main limitations include the retrospective design and the small cohort size. Also, the focus on epithelial keratinocytes without analysis of other cell types present in the subepithelial connective tissue, which may also influence the leukoplakia microenvironment.

Paper III

This study demonstrates the expression of TLR3, TLR7, TLR8, and TLR9 in OSCC cell lines (CalH3, SCC25) but also in premalignant oral epithelial cells (DOK cells). The study registered that activation of specific TLRs can regulate the expression of other intracellular TLRs, with TRIF playing a central role in downstream signaling. Importantly, TLR7 activation inhibited proliferation in premalignant cells, suggesting a tumor-suppressive function at early stages of transformation.

The findings align with previous reports from our group and others showing expression of TLR3, TLR7, TLR8, and TLR9 in OSCC[176, 232]. Interestingly, prior studies have also reported reduced TLR protein expression in OSCC compared to healthy oral mucosa [176]. Together, these results suggest that intracellular TLRs may be downregulated in OSCC, reflecting a potential tumor escape mechanism [75].

At the mRNA level, only TLR3 and TLR9 we detected in DOK and CalH3 cells, and TLR9 in SCC25 cells. Upon stimulation, CpG

ODN2006 and poly I:C downregulated TLR9 and TLR3, respectively, in DOK cells. Crosstalk between TLRs have been described previously, where co-activation can either amplify or suppress downstream responses in immune cells [233, 234]. For example, CpG ODN2006 downregulates TLR9 in plasmacytoid dendritic cells and B cells but upregulates TLR7 in plasmacytoid dendritic cells [235]. In this study, stimulation with TLR3, TLR7, or TLR9 agonists led to reduced TRIF expression in DOK cells, and poly I:C similarly downregulated TRIF in CalH3. No major effects were observed on MyD88, though earlier time points might have revealed transient changes, as poly I:C is known to upregulate TRIF within 6 hours in bronchial epithelial cells [236].

In this study, immunocytochemical staining confirmed the presence of TLR3, TLR7, TLR8, and TLR9 proteins, whereas RT-qPCR showed no detectable mRNA levels of TLR7 and TLR8 in all three cell lines. Similar differences between mRNA and protein expression were reported by Abdi et al. in multiple myeloma cells demonstrated expressions of TLR3, TLR7, TLR8, and TLR9 on the protein level in cancer cell lines, while mRNA expression levels of TLRs varied substantially between cell types [237]. The mismatch between mRNA and protein expressions may result from limited mRNA stability or regulatory processes affecting translation and post-translational protein modification [237].

The functional impact of TLR activation on cell proliferation remains complex. While poly I:C promotes apoptosis in breast cancer and OSCC cells [238, 239], CL075 enhances proliferation in HeLa cells [179], and imiquimod has been shown to inhibit proliferation in glioblastoma [240], gastric cancer [241], and OSCC [165, 242]. CpG ODN can stimulate proliferation in cholangiocarcinoma cells [243]. In the present study, however, TLRs agonists produced minimal effects on OSCC proliferation. Imiquimod inhibited proliferation only at high concentrations in CalH3, and had no effect in SCC25. By contrast, imiquimod markedly suppressed proliferation in DOK cells in a concentration-dependent manner, consistent with clinical studies showing reduced leukoplakia lesion size following topical imiquimod treatment [23, 244, 245]. Beyond immune activation [246], our results suggest that imiquimod may directly inhibit premalignant cell growth.

This study is limited by its focus on a single 24-hour time point and use of only one agonist concentration, which may have overlooked earlier or concentration-dependent effects. Another limitation is this study did not address viruses especially HPV. HPV is an established risk factor for oropharyngeal squamous cell carcinoma, but may also play a role in OSCC [8, 247]. The interaction between human papillomavirus (HPV) and TLRs shapes host antiviral immunity. Intracellular TLRs particularly TLR3 detects double-standard RNA (dsRNA), TLR7 and TLR8 recognize single-standard RNA (ssRNA), and TLR9 detect CPG motif of viral RNA and DNA to trigger antiviral signaling [248, 249]. However, HPV evades these pathways by suppressing TLR9 expression or disrupting downstream adaptors such as MyD88 [250]. Upregulated TLR4 and TLR9 in oral cancers suggest dysregulated TLR signaling drives tumor progression, while HPV may exploit these pathways to modulate immune response [177]. Understanding HPV–TLR crosstalk may identify new biomarkers and therapeutic targets for virus-associated oral malignancies

Paper IV

The study identified a panel of potential biomarkers that may distinguish malignant transformation of oral leukoplakia with a high risk of developing oral squamous cell carcinoma. The findings also support the immune system involvement in leukoplakia progression to cancer. The follow-up period, ranging from five to fifty-four months, allowed us to stratify transforming leukoplakia into short-duration (SD-leuko-ca) and long-duration (LD-leuko-ca) groups. More proteins were differentially expressed in SD-leuko-ca compared with non-transforming leukoplakia, suggesting that lesions with shorter progression time are genetically and molecularly closer to OSCC. This supports the dynamic nature of genetic and proteomic changes during malignant transformation.

Among the top biomarkers, Sortilin, collagen alpha-1(VII) chain, apolipoprotein E (ApoE), basement membrane-specific heparan sulfate proteoglycan (Perlecan) and apolipoprotein CII were downregulated in SD-leuko-ca and OSCC, whereas lanC-like protein 2, PGAM5, and ABCF2 were upregulated, showing a consistent pattern with OSCC. These findings are in line with previous reports implicating COL7A1 in multiple cancers [251-255]. In SD-leuko-ca, the upregulation of proteins

regulating gene expression, cytoplasmic translation, and ribosomal subunit biogenesis suggests enhanced protein synthesis, a hallmark of malignant progression. Increased translational activity likely supports rapid cell proliferation and metabolic reprogramming, positioning these pathways as key drivers of early transformation from leukoplakia to OSCC [256, 257]. Proteins involved in these process are kininogen-1 as a hub protein and prognostic marker in OSCC [258-262], and ApoE/ApoC-II as regulators of lipid metabolism and cancer progression [263-267]. Additional biomarkers such as elongation factor 1-delta and sortilin also showed upregulation, consistent with their roles in mesenchymal tumor biology and EGFR regulation in HNSCC [268-272].

Biomarkers distinguishing OSCC from leukoplakia included serpin B9, cytosol aminopeptidase, SUMO-related proteins, coactosin-like protein, IRF9, cleavage stimulation factor subunit 2, tapasin, and sequestome-1, all of which have documented associations with tumor progression and prognosis across cancers [273-283]. Downregulation of epithelial differentiation proteins such as keratin 10, keratin 1, cystatin-A, keratin 76 and corneodesmosin further underscores the loss of epithelial integrity during transformation, a finding consistent with previous OSCC studies [284-288].

Pathway analysis further revealed significant involvement of immune processes, with antigen processing and presentation pathways among the most significantly upregulated in OSCC compared to leukoplakia. Prior studies have shown increased infiltration of Langerhans cells, macrophages, NK cells, and T cells in dysplastic leukoplakia, as well as expansion of CD8 positive- and regulatory T cells in proliferative verrucous leukoplakia, supporting immune activation during malignant progression [230, 289-291]. We validated biomarkers displaying differentially expressed protein between groups (kininogen-1, ApoE, Perlecan, and EEF1D) were selected for immunohistochemical validation, confirming their expression in OL and OSCC tissues, although quantitative analysis was limited by sample size.

A strength of the study is that it has a prospective design with clinical data in parallel with histopathology analyses. However, the study cohort was relatively small and consisted of both dysplastic and non-dysplastic

lesions. The latter restricted subgroup analyses. Additionally, limited sample numbers precluded robust statistical assessment of immunohistochemical staining.

Taken together, the findings across the studies presented in this thesis highlight the multifactorial nature of malignant transformation in leukoplakia, with epigenetic, immunological, and proteomic factors converging to drive progression toward OSCC. Paper I demonstrated that EZH2, an epigenetic regulator, is not only a strong predictive biomarker for leukoplakia transformation, but also correlates with local T-cell infiltration, linking chromatin regulation with immune surveillance. Paper II extended this by showing that intracellular TLRs (TLR3, TLR7, TLR8, TLR9) are differentially expressed and localized in leukoplakia with and without dysplasia, suggesting that nucleocytoplasmic shuttling of TLRs may function as a regulatory switch for immune activation. Functional *in vitro* experiments in Paper III confirmed that TLR activation can modulate other TLRs through TRIF-dependent signaling, and that TLR7 agonists such as imiquimod inhibit proliferation specifically in premalignant cells, pointing to both mechanistic and therapeutic relevance. Finally, Paper IV broadened the scope by identifying proteomic signatures that discriminate leukoplakia lesions at risk of malignant transformation, highlighting both immune pathways and loss of epithelial differentiation as central processes in early carcinogenesis.

To summarize, the studies suggest that leukoplakia progression to OSCC is shaped by an interplay of epigenetic modulation, innate immune signaling, and proteomic reprogramming. The consistent involvement of immune pathways across all levels of analysis underscores the importance of the immune microenvironment in determining the risk of cancer. These findings not only provide candidate biomarkers such as EZH2, TLRs, and proteomic panels for clinical risk stratification but also suggest avenues for therapeutic intervention, for example through EZH2- and TLR-targeting agents. Future prospective studies integrating multi-omics with functional and immunological readouts will be essential to validate these markers and translate them into precision approaches for the management of leukoplakia.

6 CONCLUSIONS

Paper I

- EZH2 expression shows strong predictive potential for identifying leukoplakia at risk of malignant transformation.
- Possibly there is an interaction between EZH2-expressing keratinocytes and immune cells

Paper II

- TLR3, TLR7, TLR8, and TLR9 are consistently present in the epithelium of both leukoplakia and OSCC
- These results provide insight into the role of Toll-like receptors in early-stage oral tumorigenesis, with distinct shifts in TLR localization rather than expression levels potentially marking the transition from non-dysplastic to dysplastic leukoplakia

Paper III

- TLR3 and TLR9 gene expressions were detectable in DOK and CalH3 cell lines, but only TLR9 was expressed in SCC25
- Upon stimulation, TLR3 and TLR7 agonists reduced TRIF expression
- Protein expressions of TLR3, TLR7, TLR8, and TLR9 were detected across all three cell lines
- Imiquimod (TLR7 agonist) markedly inhibited proliferation in DOK cells
- These findings reveal distinct expression patterns and functional significance of TLRs in oral epithelial cells, suggesting their potential involvement in immune regulation and the pathogenesis of oral disease

Paper IV

- Proteomic profiling revealed progressive changes from leukoplakia to OSCC, highlighting key alterations in immune response, extracellular matrix, and epithelial differentiation pathways

- Progressive proteomic changes and altered cytoplasmic–nuclear localization of kininogen-1, Perlecan, and EEF1D may reflect functional shifts driving oral malignant transformation

Subcellular localization patterns changed with disease progression, notably with increased nuclear EZH2 in transforming leukoplakia and nuclear translocation of EEF1D and Perlecan in OSCC highlighting biomarkers of transformation. Nuclear expression of TLR7 and reduced cytoplasmic expression of TLR8 and TLR9 was observed in leukoplakia with dysplasia.

7 FUTURE PERSPECTIVES

Despite being the most prevalent oral potentially malignant disorder, leukoplakia lacks both reliable predictive biomarkers and standardized treatment strategies, creating a significant gap in clinical care. Current management relies heavily on histopathology and clinical surveillance, which do not accurately predict malignant transformation. The absence of validated molecular markers and a poor understanding of early carcinogenic events present a critical gap in clinical decision-making.

This study addresses the gap by identifying several promising molecular, proteomic and immunological changes that could enhance early detection, risk assessment, and therapeutic targeting.

- **EZH2 as a predictive marker**: The strong correlation between high EZH2 expression and transformation of leukoplakia to OSCC suggests it as a potential prognostic biomarker. Future studies should focus on validating EZH2 in larger prospective patient cohorts and exploring its role in tumor–immune interactions.

- **Targeting Toll-like receptor pathways and immunomodulation in leukoplakia:** Imiquimod, with its immune-activating properties, represents a promising non-invasive therapeutic option for high-risk leukoplakia patients who are unsuitable for surgery. Given the altered expression and localization of TLR7, TLR8, and TLR9, and their roles in early epithelial transformation, future research should focus on optimizing Imiquimod dosing and delivery while elucidating the downstream signaling dynamics of TLR7 and TLR9 in dysplastic lesions to better understand their influence on epithelial homeostasis, inflammation, and carcinogenic progression.

- **Multi-omics and subcellular dynamics in leukoplakia transformation:** A future study combining transcriptomic, and spatial analyses across stages of oral leukoplakia and OSCC could uncover early transformation events and therapeutic

targets. Special focus on subcellular localization shifts such as the nuclear translocation of proteins like EEF1D and Perlecan may further clarify mechanisms of malignant progression and support the development of localization-based biomarkers.

In summary, future research should bridge the gap between molecular discovery and clinical application by validating the findings mentioned above in prospective studies which could lead to more precise treatment strategies for patient with oral leukoplakia and OSCC.

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APPENDIX