On factors associated with development of oral squamous cell carcinoma

Fredrik Jäwert

Department of Oral and Maxillofacial Surgery,
Institute of Odontology,
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: Patient with oral leukoplakia involving the palate, buccal mucosa and gingiva (upper left). Developing oral squamous cell carcinoma in the buccal part of the oral leukoplakia (upper right). Histologic picture showing epithelial dysplasia in an oral leukoplakia (lower left). Picture from fluorescence *in situ* hybridization showing amplification of *CCND1* (green signals) in oral squamous cell carcinoma (lower right).

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fredrik.jawert@gu.se

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To all who suffer from oral cancer

On factors associated with development of oral squamous cell carcinoma

Fredrik Jäwert
Department of Oral and Maxillofacial Surgery, Institute of Odontology
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Oral squamous cell carcinoma (OSCC) has severe impacts on the affected patient's morbidity and mortality. Early detection of OSCC is of outmost importance to reduce morbidity and to improve patient survival. A significant fraction of all OSCCs is preceded by oral leukoplakia (OL). OL is clinically detectable as a more-or-less white patch in the oral mucosa. However, there is still limited knowledge regarding which patients with OL that will develop OSCC and how to best monitor and manage these patients. The overall aims of this thesis were to investigate clinical, histopathologic, genomic and epigenomic factors associated with OL progression to OSCC and to evaluate a follow-up program for these patients.

To assess if clinical follow-ups of OL patients result in early detection and high survival rate if cancer develops, a retrospective medical record and register-based study of 739 patients with OSCC was performed (**Paper I**). The results indicate that follow-ups of OL patients at an Oral and Maxillofacial Surgery - or Oral Medicine clinic results in early detection and improved survival, if the lesion transforms into cancer. Clinicopathologic features of OL that are associated with progression to OSCC were investigated in a retrospective medical record – and register-based study that included 234 patients (**Paper II**). The results showed that non-homogeneous OL progressed to OSCC to a significantly greater extent than homogeneous OLs. In addition, dysplastic OLs and OLs located at the tongue were associated with increased risk of progression to OSCC. Copy number alterations (CNAs) of known cancer driver genes were studied using fluorescence *in situ* hybridization (FISH) in OLs and OSCC (**Paper III**). CNAs in OLs that progressed to OSCC and the corresponding OSCC (N = 14) were analyzed. Comparisons were made with OLs that did not transform into OSCC (N = 14). The results showed that CNAs not only occur in OSCC but also in OLs. Some CNAs were detected somewhat more frequently in OLs that transformed into cancer. This indicates possible roles for CNAs of some genes in the development and progression of subsets of OLs. Epigenetic gene regulation mechanisms, such as DNA methylation, are involved in carcinogenesis. The epigenetic factors 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC) and ten-eleven-translocation-2 (TET2) were analyzed in OSCC (N = 15) and compared to healthy oral epithelium (N = 12) (**Paper IV**). Using immunohistochemistry, significantly lower levels of 5hmC and TET2 were detected in OSCCs compared to healthy oral epithelium.

In summary, this thesis highlights the importance of monitoring patients with OL, since it results in early detection and high survival rates, if cancer develops. In addition, we identify clinical, histopathologic, genomic and epigenomic factors that can, or have potential to, be used to identify patients with OL who are at high risk for cancer development. This knowledge may be used to identify patients who should be prioritized for regularly scheduled clinical examinations.

Keywords: oral leukoplakia, oral squamous cell carcinoma, malignant transformation, early diagnosis, genomic profiling, epigenomics

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SAMMANFATTNING PÅ SVENSKA

Cancer i munhålan utgörs till allra största del av typen skivepitelcancer och drabbar cirka 500 personer i Sverige årligen. Oral skivepitelcancer (OSEC) orsakas av förändringar i munslemhinnecellernas arvsmassa. OSEC är ofta associerat med en relativt dålig prognos, femårsöverlevnaden ligger på cirka 50%. Att diagnosticera och behandla OSEC tidigt, innan tumören blivit utbredd och spridning skett är av största betydelse för patienternas prognos, både avseende överlevnad men också för att minimera effekterna av de resttillstånd som kan ses efter genomgången behandling av en avancerad tumör. En stor del av alla OSEC föregås av ett förstadium, så kallade orala leukoplakier (OL). I Sverige, där en stor del av befolkningen går på regelbundna tandvårdsbesök, finns en möjlighet att identifiera patienter med OL och där med också patienter som löper ökad risk att utveckla OSEC. Idag har vi dock begränsad kunskap om vilka OL som utvecklas till cancer och hur vi på bästa sätt skall ta hand om dessa patienter. Syftet med studierna i avhandling var att studera olika kliniska-, histologiska-, och genomiska faktorer hos OL som kan identifiera de patienter som löper en hög risk att utveckla OSEC, samt att utvärdera om regelbunden klinisk uppföljning av dessa patienter resulterar i en bättre prognos i de fall cancer utvecklas.

För att studera om patienter med OL som följs upp på en specialistklinik för käkkirurgi eller oral medicin/sjukhustandvård diagnosticeras tidigt och har bättre 5-årsöverlevnad om cancer utvecklas utfördes en retrospektiv studie där 739 patienter med OSEC inkluderades (**Studie I**). Resultaten visar att patienter med OL som följs upp har en bättre 5-årsöverlevnad och diagnosticeras i ett tidigare skede om cancer utvecklas. I **Studie II** studerades kliniska- och histologiska faktorer av OL och dess association till cancerutveckling. Frågeställningen studerades retrospektivt med 234 inkluderade patienter. Informationen i både studie I och II baserades på patientjournaler och det regionala cancerregistret. Resultateten visade att icke-homogena OL utvecklades till cancer i 15 gånger så hög utsträckning som homogena OL. Även OL som uppvisade cellförändringar vid den mikroskopiska undersökningen, samt OL lokaliserade till tungan var associerade med en ökad cancerutveckling. I **Studie III** undersöktes om det fanns ett ökat antal genkopior av vissa gener som gynnar cancerutveckling (onkogener), samt förlust av genkopior i en gen som har en negativ regulatorisk funktion på celldelning och cancerutveckling (tumörsuppressorgen) i OL och OSEC. Genvariationerna studerades med fluorescerande *in situ*-hybridisering (FISH) i 14 OL som utvecklades till cancer, de efterföljande cancrarna samt i 14 OL som inte transformerades till OSEC. Ett ökat antal genkopior av onkogener, och förlust av tumörsuppresorgenen upptäcktes i vissa av cancrarna men även i vissa OL. Vissa variationer i genkopior påträffades i något högre omfattning i OL som utvecklades till cancer jämfört med de OL som inte genomgick cancerutveckling. Resultaten från studien indikerar att förändringar i antal kopior av dess gener kan vara involverade i uppkomsten av vissa OL och i övergången till OSEC i vissa OL. I **Studie IV** studerades faktorer involverade i den epigenetiska genregleringen: 5-metylcytosin (5mC), 5-hydroxymetylcytosin och ten-eleven-translocation 2 enzymet (TET2) i vävnadspreparat från 15 OSEC och 12 friska munslemhinnor. Immunhistokemisk analys visade kraftigt reducerat antal positivt infärgade cellkärnor av 5hmC i cancer jämfört med den friska slemhinnan. Även uttrycket av TET2 var signifikant reducerat i cancer jämfört de friska munslemhinnorna.

Sammanfattningsvis visar resultaten från avhandlingen att uppföljning av patienter med OL på en specialistklinik resulterar i tidig upptäckt och en bättre 5-årsöverlevnad om cancer utvecklas. I studierna identifierades även kliniska-, histopatologiska-, genomiska- och epigenomiska faktorer som är eller har potential att vara användbara för att identifiera OL patienter med hög risk att utveckla cancer. Faktorerna kan vara användbara för att identifiera de patienter som bör prioriteras med täta kliniska kontroller.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Jäwert F, Nyman J, Olsson E, Adok C, Helmersson M, Öhman J. Regular clinical follow-up of oral potentially malignant disorders results in improved survival for patients who develop oral cancer. Oral Oncol. 2021;121:105469.

II. Jäwert F, Pettersson H, Jagefeldt E, Holmberg E, Kjeller G, Öhman J. Clinicopathologic factors associated with malignant transformation of oral leukoplakias: a retrospective cohort study. Int J Oral Maxillofac Surg. 2021;50(11):1422-1428.

III. Jäwert F, Fehr A, Öhman J, Stenman G, Kjeller G. Copy number profiling reveals recurrent oncogenic events in oral leukoplakias. *In manuscript* 2021.

IV. Jäwert F, Hasséus B, Kjeller G, Magnusson B, Sand L, Larsson L. Loss of 5-hydroxymethylcytosine and TET2 in oral squamous cell carcinoma. Anticancer Res. 2013;33(10):4325-8.

CONTENT

ABBREVIATIONS

CCND1	Cyclin D1
CDK	Cyclin-dependent kinas
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CI	Confidence interval
CIS	Carcinoma *in situ*
CNA	Copy number alteration
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
FFPE	Formalin-fixed and paraffin-embedded
FISH	Fluorescence *in situ* hybridization
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	Hazard ratio
ICD	International classification of diseases
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
MT	Malignant transformation
MTR	Malignant transformation rate
OD	Oral dysplasia
OL	Oral leukoplakia
OM	Oral Medicine
OMFS	Oral and Maxillofacial Surgery
OPMD	Oral potentially malignant disorder
OSCC	Oral squamous cell carcinoma
QoL	Quality of life
TET	Ten-eleven translocation
TMA	Tissue microarray
TNM	Tumor, nodes, metastasis (Staging system)
UICC	Union for International Cancer Control
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine

1 INTRODUCTION

Cancer is a leading cause of death worldwide. International Agency for Research on Cancer estimated that in 2020 there were 19.3 million new cases of cancer, and almost 10 million cancer-related deaths. In Sweden, there are more than 60,000 new cancer cases annually. It is said that every third person in Sweden develops cancer, whereas everyone, in some way, is affected by cancer. Cancer incidence and cancer-related mortality rates are, in general, increasing around the world. An aging and growing population is one reason, and increased exposure to risk factors is another.

Cancer of the oral cavity has a strong impact on the affected patient's morbidity and mortality. Early detection of oral cancer is of importance for the patient's survival and quality of life (QoL), as well as in terms of treatment costs. A significant fraction of all cancers of the oral cavity is preceded by so-called 'oral potentially malignant disorders' (OPMDs). OPMDs are lesions or conditions in the oral mucosa that are associated with an increased risk of cancer development. Oral leukoplakia (OL) is one of the most common OPMDs. OL is clinically detectable as a more-or-less white patch in the mucosa. Since the majority of persons in Sweden visit a dental care facility on a regular basis, there are good opportunities to identify patients with OL. Thus, we can identify persons who have an increased risk of oral cancer development. However, we still have limited knowledge regarding which patients with OL will develop oral cancer and how to best treat and take care of these patients. This thesis focuses on OLs and factors associated with oral cancer development, to acquire new knowledge on how to monitor and risk-stratify these patients, and thereby facilitate the early detection of oral cancer. This would lead to improved prognosis and survival rates, with reduced impact on the QoL of the patients.

1.1 ORAL CANCER

Among the malignant tumors of the oral cavity, oral squamous cell carcinoma (OSCC) (Figure 1) is the most common, accounting for more than 90% of all cases (1). OSCC has significant impact on both morbidity and mortality. The tumor originates from the epithelial cells lining the oral mucosa (1), and are anatomically categorized into: *floor of the mouth, hard palate, maxillary gingiva, mandibular gingiva, tongue* (anterior 2/3), *and buccal mucosa* (2). OSCC is included in the head and neck squamous cell carcinoma (HNSCC) group, which also includes carcinomas of the nasal cavity, paranasal sinuses, nasopharynx, oropharynx, hypopharynx and larynx (1).

In 2020, the estimated global incidence of OSCC, including the lips, was 377,713 new cases. OSCC-related deaths were estimated at 177,757 (3). OSCC was the sixteenth most commonly reported cancer globally 2020, and accounted for 2.1% of all cancer cases (4). There are significant geographic differences in OSCC incidence in the world. In India, Afghanistan, Pakistan, Papua New Guinea and Sri Lanka, OSCC is the most common cancer among males (3). In India and Pakistan, OSCC is also the most-common cancer-related cause of death in men. India, Sri Lanka and Papua New Guinea have the highest reported incidences of OSCC in both genders world-wide. OSCC is, in general, more common in males than in females (1). In Sweden, there were 454 new cases of OSCCs reported in 2019. Of these, 237 were male (52.2%), with a median age at diagnosis of 69 years. The remaining 217 (48.8%) were females, with a median age at diagnosis of 74 years (5).

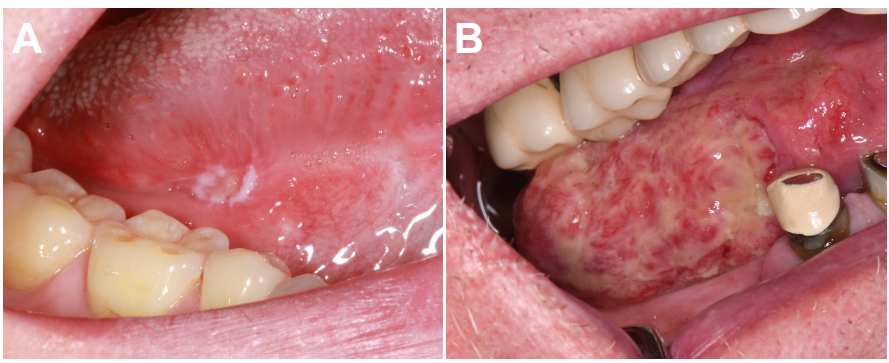

Figure 1. *Oral squamous cell carcinoma at right lateral border of tongue. (A) Early stage - and (B) an advanced tumor stage.*

1.2 THE IMPORTANCE OF EARLY DETECTION OF ORAL CANCER

To detect and treat OSCC at an early stage of the disease is of significant importance for several reasons. Here, early detection is discussed in relation to: *prognosis*; *extent of treatment*; *quality of life*; and *treatment costs*.

The staging and grading of OSCC is based on the TNM staging system of the Union for International Cancer Control (UICC) (2) and the American Joint Committee on Cancer (AJCC). TNM is an anatomical staging system that describes the tumor burden in the body. The 'T' in TNM describes the primary tumor in terms of size and invasiveness in the surrounding tissues. The 'N' describes the metastatic status of the loco-regional lymph nodes; and the 'M' describes distant metastasis. Based on the TNM system, the disease is assigned to one of four major stages (I–IV). Stage I represent the least-advanced stage, with a small primary tumor and no evidence of spread. Stage IV represent the most-advanced disease stage, represented as metastatic disease and/or where the primary tumor has invaded the cortical bone, the deep muscles of the tongue, the facial skin or the maxillary sinus (2).

Prognosis

The overall 5-year survival rate for patients with OSCC is approximately in the range of 50–60% (6-9). Early detection is of the utmost importance for the prognosis, with the 5-year survival rate decreasing from over 80% to around 30% when detection is made at a late stage (IV) compared to an early stage (I) (7). Spread of the disease has a major negative impact on the prognosis for the patient. OSCC metastasizes mainly to the cervical lymph nodes, and this is reported to reduce the survival rate by approximately 50%, as compared to a non-metastatic OSCC (10).

Treatment of OSCC

Surgery is the primary treatment strategy for OSCCs. The surgical treatment consists of complete excision of the tumor with adequate surgical margins, and eventual neck dissection with extirpation of the cervical lymph nodes (11). The treatment modality is mainly based on the staging system (2). Treatment decisions are with advantage made with the support of Multidisciplinary Tumor Boards (12). Early-stage OSCC, i.e., small primary tumors without evidence of spread, are mainly treated by local surgical excision, sometimes with complementary elective neck dissection (11). Advanced-stage OSCCs, i.e., large primary tumors with spreading to the cervical lymph nodes, are

treated more aggressively with surgery that includes removal of the regional lymph nodes (Figure 2). A combination with adjuvant therapy, such as postoperative radiotherapy (in some cases, also with chemotherapy), is then administered after surgery (11).

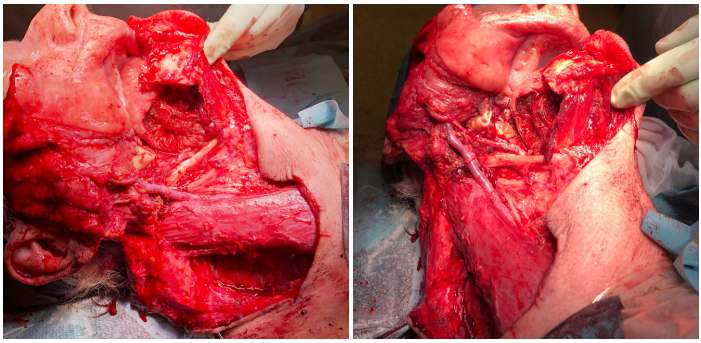

Figure 2. *Surgical resection of a late stage gingivomandibular squamous cell carcinoma. The pictures show status after tumor resection and neck dissection, but before insertion of a free vascularized fibula flap.*

Quality of life

OSCC and the treatment for OSCC have significant impacts on the affected patient's QoL. WHO defines QoL as: "*an individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns"* (13). Early detection is of great importance in terms of the eventual QoL for OSCC patients. In a study conducted by Rogers et al (14) involving 561 patients who received primary treatment with surgery for their oral or oropharyngeal cancer, QoL factors were evaluated. Patients with early-stage carcinomas, T1 or T2, without need for adjuvant radiotherapy and without need for advanced reconstructive surgery (such as free flaps), had the highest reported QoL (Figure 3A). In contrast, patients who received adjuvant radiotherapy had the lowest reported QoL (Figure 3B). Other studies have as well reported statistically significant lower QoL in OSCC patient receiving adjuvant radiotherapy (15, 16). Radiotherapy to the head and neck region has significant side-effects, which included oral mucositis, hyposalivation, dysphagia and osteoradionecrosis of the jaws (17). Free flaps are used to reconstruct the most advanced cases, and have also been reported to have a negative impact on QoL (18). This is probably not only due to the flap per se (19), but rather to the advanced disease.

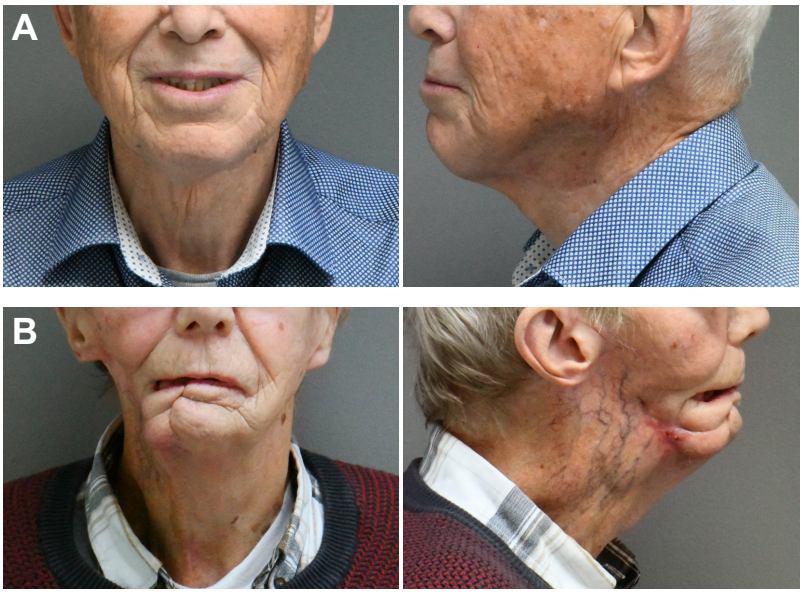

Figure 3. *Physical appearance of two patients surgically treated due to gingivomandibular squamous cell carcinomas. Patient A, detected early, with a small primary tumor at the left mandibular gingiva without signs of spread. The patient was treated by local tumor resection and an elective neck dissection. Patient B, detected late with an advanced carcinoma at the right mandibular gingiva, with spread to the cervical lymph nodes. The patient was treated with surgery at both tumor site and neck and subsequently with adjuvant radiotherapy. Later, he was also affected by osteoradionecrosis.*

Treatment costs

In addition to ensuring higher survival and having a weaker impact of QoL, early detection of OSCC results in lower treatment costs (20). Zavras and coworkers (21) reported statistically significant higher treatment costs for advanced-stage (III, IV) OSCC than for early-stage (I, II) OSCC. An advanced stage of disease was also associated with a statistically significant longer duration of hospitalization. Jacobson et al (22) showed that multiple treatment modalities for OSCC were twice as costly as single modality treatments. The authors proposed that treatment of advanced-stage OSCC with all treatment modalities combined entailed one of the highest treatment costs for all cancers in the US. The authors concluded that since multimodal treatments are applied owing to the presence in the patients of advanced-stage carcinomas, early detection is of the utmost importance when considering treatment cost reductions.

1.3 ORAL POTENTIALLY MALIGNANT DISORDERS

Oral potentially malignant disorders (OPMDs) represent a group of oral mucosal lesions and conditions with different risks of undergoing malignant transformation (MT) (23). The majority of OPMD cases do not proceed to OSCC, although they have an increased risk of OSCC development compared to healthy oral mucosa (24). Conversely, in a significant percentage of cases, OSCC is preceded by an OPMD. It is reported that approximately 50% of patients with OSCC have a co-existing OPMD-like lesion (25). OPMD comprises different disorders of the mucosa with different malignant transformation rates (MTRs) and prevalence. In the latest consensus report from the WHO Collaborating Centre for Oral Cancer (23), the following diagnoses were considered as OPMDs:

- Oral leukoplakia
- Erythroplakia
- Proliferative verrucous leukoplakia
- Oral lichen planus
- Oral submucous fibrosis
- Palatal lesions in reverse smokers
- Lupus erythematosus
- Dyskeratosis congenita
- Oral lichenoid lesions
- Oral manifestations of graft-versus-host disease

In this thesis, the main focus is on OLs. However, in **Paper I**, other OPMDs were also included.

1.3.1 ORAL LEUKOPLAKIA (OL)

OL is defined as "*A predominantly white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer*" (26) (Figure 4). The diagnosis involves the exclusion of other diagnoses from both the clinical and histopathologic aspects (26). OL is one of the more common OPMD diagnoses, and has an estimated global prevalence of 1.5%–4.1% (27, 28). The MTR of OL ranges from 0.13% to 34.0% in different studies (29). In a meta-analysis published in 2020 by Iocca et al, which included 17,830 patients with OL, an MTR of 9.5% was reported with an estimated yearly MTR of 1.56% (24). In a meta-analysis published this year, 2021, an MTR of 9.8% (95% CI: 7.9—11.7%) was reported, based 16,604 patients with OL (30).

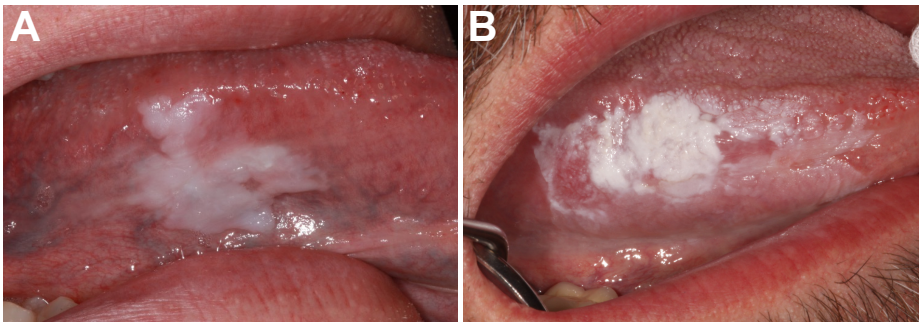

Figure 4. *Oral leukoplakias at the right lateral border of tongue. (A) Homogenous oral leukoplakia and (B) non-homogenous oral leukoplakia.*

1.3.2 CLINICOPATHOLOGIC ASPECTS OF OL

Clinically, OL is divided into homogeneous and non-homogeneous subgroups. A homogeneous OL is well-demarcated, with a uniformly white, plaque-like appearance and flat surface (Figure 4A) (23). A non-homogeneous OL has a more speckled appearance with irregular red and white areas and/or with a more nodular, speckled or verrucous surface (Figure 4B) (23). Non-homogeneous OL is reported to have a higher MTR than homogeneous OL (30-34) and is considered to be a high-risk lesion. Other clinical features that have been associated with an increased MTR include: OL located on the tongue (32, 35); large OLs (34), i.e., those having an area >200 mm^2 (31) or largest diameter ≥4 cm (36); OLs in females (30, 33); and OLs in non-smokers (33, 37).

In the histopathologic examination of OL, the major aim is to exclude malignancy, while another aim is to evaluate the presence of dysplasia. Dysplastic OLs are associated with an increased risk of MT compared to non-dysplastic OLs (30, 32, 35, 38). An assessment of dysplasia is based on cytologic and architectural changes in the epithelium (1, 39). Among the cytologic changes encountered are: atypical mitosis; abnormal cellular and nuclear sizes and shapes; increased nuclear-to-cytoplasmic ratio; hyperchromasia; and increased number and size of the nucleoli (1). Architectural changes to the epithelium, such as irregular epithelial stratification, loss of polarity of basal cells, drop-shaped rete ridges, abnormal superficial mitosis, increased number of mitotic cells, premature keratinization, keratin pearls in the rete ridges, and loss of cell cohesion (1). Dysplasia is categorized as mild, moderate or severe. In the grading of dysplasia, the epithelium height is divided into the basal-, middle- and upper-thirds. In mild dysplasia, cytologic and architectural changes are present in the basal-third. For moderate dysplasia, the changes have to be evident up to the middle-third. When the upper-third is affected, the dysplasia is considered to be severe (1). However, the cytologic aspects can increase the grade of dysplasia irrespective of occurrence in the corresponding epithelial level. In the current edition of the *WHO Classification of Head and Neck Tumours* (4th edition), the MTRs are estimated at 6%, 18% and 39% for mild moderate, and severe dysplasia, respectively (1). However, the assessment as to whether dysplasia is present and the grading of dysplasia are subjective, with significant variability seen for the inter- and intra-observer reliabilities (40). In an attempt to improve the inter- and intra-observer reliabilities, a binary system has been suggested, consisting of a low-grade and a high-grade dysplasia (1). The cut-off between low-grade and high-grade dysplasia is estimated to lie within the span of moderate dysplasia in the traditional grading system (1). Kujan and coworkers have proposed that when four or more architectural changes and

five or more cytologic changes are observed, this can be considered as a high-risk dysplasia (41). Nankivell and coworkers (42) have found increased sensitivity when four or more cytologic alterations are used, in addition to the four architectural changes. In a recently published systematic review and meta-analysis, it was concluded that there is, so far, no evidence to suggest that these binary systems are preferable over the WHO dysplasia grading system for the prediction of MT (43). The binary system has, as mentioned earlier, higher inter- and intra-observer reliabilities. Dysplasia remains the gold standard assessment for evaluating the risk of MT of OL.

1.3.3 TREATMENT AND CARE OF PATIENTS WITH OL

The overall treatment goal for patients with OL is to prevent MT. The preventive effects of OL treatments are discussed in the literature (44-49). While it is intuitive to believe that surgical removal of OLs saves the patient from subsequent MT in the operated area, the results of some retrospective studies indicate that surgically removed OLs do not have a lower MTR than OLs that are left without intervention (31, 36). There are even results showing that OLs treated with surgery have an increased rate of transformation (31). In addition, the recurrence rate of OL is reported to be high after surgical excision (36, 50). To date, there have been no conclusive published randomized controlled trails evaluating the preventive effects of surgical treatments on the MT of OL. In a recently published retrospective study, Gilvetti and coworkers reported a lower MTR for patients with oral dysplasia (OD) treated with surgery compared to ODs that were left intact: 4/14 (28.6%) non-treated ODs proceeded to OSCC compared to 13/106 (12.5%) of the surgically treated ODs (51). A lower MTR for treated ODs than for non-treated ODs is consistent with the results of the systematic review and meta-analysis, in which non-treated ODs had an MTR of 14.6% and treated ODs had an MTR of 5.4% (52). In addition, Gilvetti and coworkers showed that both the recurrence rate and MTR correlated with positive surgical margins (51). Inadequate surgical margins are regarded as a contributory factor to the high recurrence rate of OL (44). Furthermore, the clinical border of the lesion shows limited consistency with the histopathologic circumscription, and is even less consistent with the area of genetically altered cells (53-55). The term *Field cancerization* was introduced by Slaughter in 1953 to describe patients who have multiple, recurrent OSCCs in an area of mucosa with atypical histology (56). Today, this is attributed to cells within clinically “healthy” mucosa that harbor genomic alterations (55). *Field cancerization* may explain the recurrence of OSCC after surgical treatment, as well as ´second primary´ OSCCs and recurrent OLs.

Non-surgical treatment strategies have also been investigated with respect to the prevention of MT of OLs. In 2016, Lodi et al (47) published a systematic review dealing with the prevention of MT of the OL in which they concluded that there is no evidence that either surgical or non-surgical treatment methods prevent MT of the OL. However, one should bear in mind that even if surgical removal of OLs is not considered as a preventative measure for future MT, there is a distinct benefit associated with enabling histologic examination of the whole lesion (57).

In 2009, van der Waal published a review of recommendations regarding the management of patients with OL (58). The general recommendation was to, if

possible, remove all OLs, regardless of whether the incisional biopsy shows the presence or absence of dysplasia. If excision is not feasible, the recommendation was to map the lesion using multiple incisional biopsies acquired from the different reaction patterns of the lesion (58, 59). In addition, a more-or-less life-long follow-up of patients with OL is proposed, regardless of whether the OL is removed or not. For dysplastic lesions, a follow-up interval of at least 3 months is suggested. In cases with the absence of dysplasia, follow-up every 6 months is recommended. The purpose of continuous follow-ups is, of course, to detect at an early stage if MT of the OSCC occurs.

An important task for healthcare providers as part of the care of patients with OL is to educate and motivate. Lifestyle changes that take into consideration OSCC risk factors, such as the promotion of smoking cessation and restricted alcohol consumption, are crucial in minimizing the risk of OSCC development.

1.4 ENVIRONMENTAL AND BEHAVI(ORAL) RISK FACTORS FOR OSCC AND OL

Smoking and smokeless tobacco usage are the major carcinogenic, lifestyle-related risk factors for accumulation of the genomic alterations underlying OSCC development (60 - 62). Betel quid chewing is a distinct and potent risk factor for OSCC development (63), and it explains the high incidence rates of OSCC in countries such as India, Sri Lanka and Papua New Guinea. Alcohol over-consumption is also a risk factor for OSCC development (64), and in combination with smoking it represents a significantly higher risk (65). Alcohol seems to potentiate the carcinogenic effect of smoking. Infection with human papillomavirus (HPV) is a well-known risk factor for the development of carcinomas in the cervix uteri, as well as in the oropharynx (1). The association between HPV infection and OSCC has been thoroughly studied, although the association seems to be less clear-cut than the linkages to cervical cancer and oropharyngeal cancer. Studies have reported a wide range of HPV prevalence rates in patients with OSCC. Syrjänen and Syrjänen, in summarizing five meta-analyses published after 2010, reported an HPV prevalence of 13–58% (66). That review also showed distinct geographic differences. In a multicenter, case-controlled study conducted by Herrero and coworkers, 3.9% of 766 PCR-validated OSCC specimens were found to be HPV-positive (67). The potentially causal role of HPV in OSCC is debated extensively within the scientific community.

While the etiology of OL is not fully understood. Genomic alterations are involved. Smoking is a major lifestyle-related risk factor for OL development (68). In addition to smoking, betel quid chewing and alcohol consumption have been identified as risk factors in an Asian population (69). However, alcohol consumption as a risk factor for OL is unclear, given that other studies were unable to identify an increased risk for OL development (68). HPV is also discussed as a risk factor for OL. However, the causal effects of HPV for OL are widely debated in the literature. The association seems to be even less clear than the association described between OSCC and HPV (70-72).

1.5 CANCER GENOMICS

Cancer is characterized by uncontrolled and abnormal cell proliferation, resulting in the formation of a tumor, and the cells gaining the ability to invade adjacent tissues and spread to other organs. The mechanism of carcinogenesis is alterations in our genetic material, the deoxyribonucleic acid (DNA) (73, 74). Tumor development is a multistep process that requires several alterations in different genes. In total, there are several hundred known genes that could be involved in tumor development (75). Three types of genes are mainly involved: oncogenes, tumor-suppressor genes, and DNA repair genes (76).

In the highly cited paper *The hallmarks of cancer*, Hanahan and Weinberg describe how the tumor cells acquire new biologic properties that are characteristic of tumor cells, through alterations in several different genes (73). The new biologic properties are: *sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis,* and *activating invasion and metastasis*. These respective properties are briefly described below. In an updated version of the paper, *Hallmarks of cancer: next generation,* new biological capabilities were added that include; *evading immune destruction* and *reprograming energy metabolism* (74).

1.5.1 HALLMARKS OF CANCER

Sustaining proliferative signaling

Under normal healthy circumstances, the cell division process is well-controlled, resulting in homeostasis of cell numbers and tissue architecture. In contrast, tumor cells have acquired a chronic mode of mitotic signaling, resulting in uncontrolled cellular proliferation (73, 74). Proto-oncogenes are normal genes in our genome that encode proteins that function in cell growth, division and survival. If the proto-oncogene is affected by a genomic alteration that results in the increased expression or increased activity of the protein it is activated to become an oncogene. Activation of proto-oncogenes to oncogenes is the underlying mechanism through which the tumor cells experience chronic proliferative stimulation. Well-known oncogenes in HNSCC include: *EGFR* (epidermal growth factor receptor), *PIK3CA, MYC* and *CCND1* (cyclin D1) (77, 78).

Evading growth suppressors

Growth suppressors are proteins in the cell that negatively regulate cell proliferation. Growth suppressors ensure that the cells only undergo mitosis when the circumstances for proliferation are 'appropriate', such as during wound healing, normal cellular turnover or normal growth. The growth suppressors, which are activated by both intrinsic and extrinsic factors, arrest the cell cycle or induce apoptosis. Cancer cells must evade growth suppressors in order to have unrestricted mitotic activity (73, 74). Tumor suppressor genes are genes in our genome that encode proteins that negatively control cell proliferation, i.e., they have an important anti-tumorigenesis function. In contrast to the activation of oncogenes, which represents gain of function, the loss of function of tumor suppressor genes requires alteration of both setups of the gene. Commonly inactivated tumor suppressor genes in HNSCC include *TP53* and *CDKN2A* (77, 78). The *TP53* gene encodes the p53 protein and is called the *"Guardian of the genome"*. The p53 protein is activated by DNA damage, and the activated p53 protein arrests the cell cycle at the G1/S restriction point, activates DNA repair proteins, activates cellular senescence, and even initiates apoptosis. Overall, 70% of the HNSCCs in cBioPortal database (www.cbioportal.org) shows mutations in the *TP53* gene (77, 78), making it the most common genomic alteration in HNSCC. *CDKN2A* is a central tumor suppressor gene that encodes the p16 and p14ARF proteins, which act as tumor suppressor proteins. The p16 protein inhibits CDK4 and CDK6, resulting in arrest at the G1/S cell cycle checkpoint. The p14ARF protein negatively regulates Mdm2, which is an oncogene that in turn negatively regulates p53.

Resisting cell death

Programed cell death, apoptosis, acts as a self-defense mechanism against tumor development. Apoptosis is initiated in specific circumstances, such as major DNA damage or increased oncogenic signaling. However, the tumor cells can circumvent this restriction, which the consequence that cells with considerable levels of DNA damage and high-level oncogenic signaling continue to grow (73, 74). The biologic capability to resist cell death is acquired through the over-activation of anti-apoptotic factors and the down-regulation of pro-apoptotic factors (73, 74, 79, 80).

Enabling replicative immortality

Most of the cell lineages in humans have a limited number of cell cycles. Cancer cells have acquired an unlimited replicative potential, which underlies their potential to produce a macroscopic tumor. Telomeres are repetitive sequences of non-coding DNA at the ends of the chromosomes. The telomeres protect the chromosome from nucleolytic degradation and end-to-end fusion. At each cell cycle, the telomeric DNA is shortened, eventually reaching a critical limited length that is non-protective. At this level, the cell is either entering senescence, which is a viable state in which the cell is unable to proliferate, or undergoing apoptosis. Telomerase is a DNA polymerase that adds DNA sequences to the telomeres. Under normal circumstances, telomerase is active in germline cells and some hematopoietic cells but is not active or is active at only low levels in somatic cells. Cancer cells reactivate their telomerase activity (73, 74, 81).

Inducing angiogenesis

For the tumor cells to be able to grow and generate a macroscopic tumor, they need oxygen and nutrients, as well as the ability to eliminate waste products. In order to establish these circumstances, the tumor cells induce angiogenesis. Angiogenesis, which is the process of sprouting of new blood vessels, occurs for example during wound healing and in the female reproductive system. Tumor cells induce angiogenesis. Vascular endothelial growth factor (VEGF) binds to endothelial cells and stimulates proliferation. Upregulation of *VEGF* gene expression can occur as a response to hypoxia, but also as a result of oncogene signaling (82, 83).

Activating invasion and metastasis

The squamous cells in the oral epithelium are tightly attached to each other

through cell-to-cell adhesion, and the basal epithelial cells are also attached to the extracellular matrix (ECM) in the form of the basement membrane. E-cadherin mediates cell-to-cell adhesion, and hemidesmosomes anchors to the basement membrane. This gives the squamous epithelium its characteristic sheet architecture. For the incipient cancer cells to spread and colonize distant tissues, they must detach from the neighboring cells, pass through the basement membrane, invade the lymph vessels, metastasize in the lymph nodes, migrate into the blood vessels and exit the vessels to form micro-metastases, for subsequent formation of a macro-metastasis. This process is referred to as 'the invasion and metastasis cascade' (73, 74).
Among the factors involved in the invasion and metastasis linked to OSCC are the downregulation of E-cadherin (84) and the overexpression of matrix metalloproteinases, which are enzymes that modulate the ECM (85).

1.5.2 GENOMIC ALTERATIONS

There are different types of genomic alterations, including mutations, copy number alterations and chromosomal rearrangements (86). In this thesis, copy number alterations (CNAs), which consist of amplifications/gains and losses of genes or chromosomal segments, are investigated. Copy number gains/amplification is a mechanism to activate oncogenes, and copy number loss is a mechanism to inactivate tumor suppressor genes.

1.5.3 EPIGENOMIC ALTERATIONS

Epigenetics is the study of heritable changes affecting gene expression that do not involve changes in the DNA sequences (87). These changes are affected by lifestyle behaviors and by environmental factors. Since alterations to the epigenome affect gene expression, it may be involved in the acquisition of the biologic capabilities of tumor cells. Epigenetic mechanisms regulate gene expression, for example, by changing the configuration and structure of the chromatin or chromatin-associated proteins and, thereby, the abilities of transcription factors to bind to the DNA sequences (87). Methylation of the nucleotide cytosine, to 5-methylcytosine (5mC), is a well-known epigenetic regulation mechanism. The methyl group in 5mC interferes with the ability of the transcription factor to bind to its target DNA, thereby modulating gene expression (88). Alterations to the DNA methylation pattern affect the normal pattern of gene expression and are involved in tumorigenesis. For example, hypermethylation of the promotor region in a tumor suppressor gene can silence the gene. 5mC can be further modified to 5-hydroxymethylcytosine (5hmC), in a reaction that is catalyzed by the ten-eleven-translocation (TET) family of enzymes (87). 5hmC is an intermediate in the DNA demethylation process (89). Reduced levels of 5hmC have been observed in various cancers (90 – 94).

1.5.4 GENOMIC ASPECTS OF OL AND MALIGNANT TRANSFORMATION

Although the etiology of OL is not fully understood, genomic alterations are involved and implicated as the driving force for the MT of OL. MT is a multistep process that requires alterations in several different genes. Numerous different genomic alterations have been investigated in OL and in the MT of OL (95–100). Figure 5 shows the model of Dionne and coworkers that illustrates the multistep process of oral carcinogenesis and some the genes that are involved (101). As in OSCC, mutations in *TP53* have been intensely studied, and are associated with the MT of OLs (95, 96, 97). Recently published systematic reviews have summarized the current knowledge of biomarkers in terms of predicting MT of OL. The majority of the included biomarkers were different genomic alterations. The conclusion of these systematic reviews is that science still is in the discovery phase and that, to date, there are no reliable biomarkers for the prediction of MT (95, 102). OSCC and OLs are heterogeneous tumors/lesions in terms of their genomic profiles (77, 78, 96), which complicates the detection and development of reliable biomarkers.

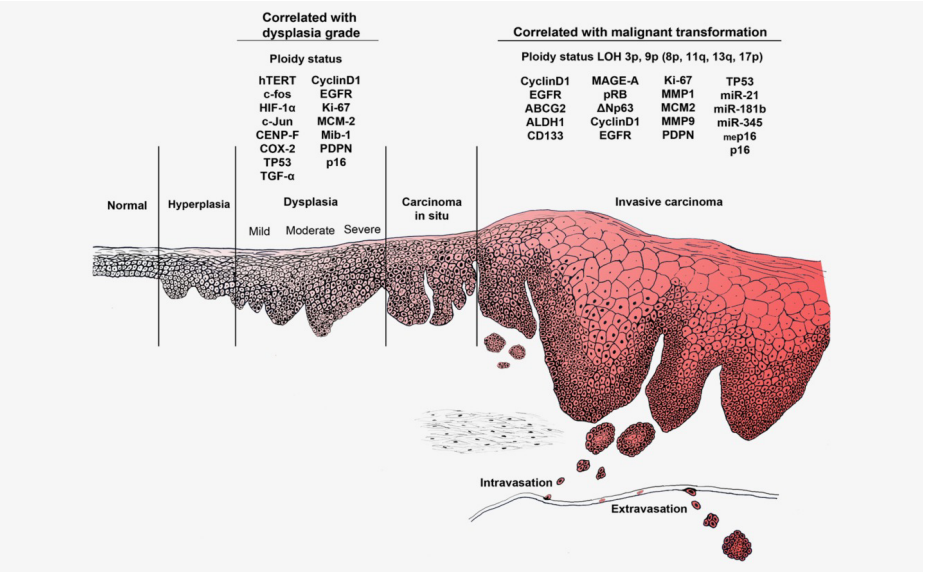

Figure 5. *Multistep process of oral carcinogensesis including some of the altered genes that are involved. Reprinted with permission from International Journal of Cancer, WILEY. Dionne KR, Warnakulasuriya S, Zain RB, Cheong SC. Potentially malignant disorders of the oral cavity: current practice and future directions in the clinic and laboratory. Int J Cancer. 2015 Feb 1;136(3):503-15. doi: 10.1002/ijc.28754. Epub 2014 Feb 11. PMID: 24482244.*

In this thesis, CNAs in OLs and OSCCs are investigated. CNAs have previously been studied in OPMDs, and in the MT of OPMDs. Among the CNAs studied in OPMDs are the oncogenes *EGFR* and *CCND1* (103-105). Previous studies have shown that OPMDs that progress to carcinoma in situ (CIS) and/or OSCC have an increased copy number of *EGFR* compared to those that do not progress (103, 104). In the Erlotinib Prevention of Oral Cancer (EPOC) trial, William and coworker reported that OPMDs with an increased copy number of *EGFR* had a significantly lower oral cancer-free survival (OCFS) rate (105). CNAs of *CCND1* were investigated by Poh and coworkers (103). Overall, 21/22 (96%) of OPMDs that progressed to CIS or OSCC showed an increased copy number of *CCND1*. Among the OPMDs that did not progress to CIS or OSCC, 4/13 (31%) showed a copy number gain for *CCND1*. Rosin and coworkers studied loss of heterozygosity (LOH) in chromosomes 3p, 4q, 8p, 9p, 11q, 13q, and 17p in progressing and non-progressing OPMDs (106). LOH of 3p and 9p was significantly more common in OPMDs that progressed to CIS or OSCC (106). The significant LOH of 3p and 9p in progressing OPMDs was verified in a larger prospective setup (107). A higher percentage of LOH of chromosome 9p in progressing OLs compared to non-progressing OLs has also been reported in other studies (108).

Chromosomal regions that are frequently affected by CNAs in patients with HNSCC include losses in chromosomes 3p and 9p and gains in chromosomes 3q, 5p, 7p, and 8q (109-111). Loss of the tumor suppressor gene *CDKN2A* in chromosome 9p21.3 is the most-prevalent, gene-specific CNA in HNSCCs. Overall, 30% of HNSCC cases show loss of *CDKN2A* (77, 78). Gains of *CCND1, FGF3* and *FGF4* in 11q13.3 occur in more than 20% of HNSCC cases. Additional frequent gene specific gains detected in HNSCC are *PIC3CA* and *SOX2* in 3q26, *TP63* in 3q28, and *MAP3K13* in 3q27, which occur in approximately 15% of cases. Gains of *EGFR* in 7p11.2 and *MYC* in 8q24.21 represent other relatively common, gene-specific CNAs and occur in approximately 10% of HNSCC cases (77, 78).

2 AIMS

The overall aims of this thesis were to identify risk factors of OL that predict MT and to evaluate a follow-up program for these patients. The future intention is to facilitate the early detection of oral cancer, and thereby improve survival rates and reduced the impact on the QoL of the affected patients.

Paper I

Investigates whether patients with OPMD who undergo continuous clinical examinations have higher 5-year survival rates and lower tumor burdens when MT occurs, as compared to patients with OPMD without follow-ups and other patients with OSCC.

Paper II

Describes the clinicopathologic features of OLs that are associated with MT. The primary clinicopathologic factor studied was clinical subtype of OL, homogeneous or non-homogenous.

Paper III

Characterizes differences in gene-specific gains of some oncogenes and loss of a specific tumor suppressor gene in OSCCs, OLs progressing to OSCC and in OLs not progressing to OSCC.

Paper IV

Characterizes differences in levels of 5-methylcytosine, 5-hydroxymethylcytosine and ten-eleven-translocation-2 in OSCC compared to healthy oral epithelium.

3 PATIENTS AND METHODS

3.1 PAPER I

Patients

Patients with OSCC were identified through searches using the protocol from the Multidisciplinary Treatment Conference of Head and Neck Malignancies, Sahlgrenska University Hospital, Gothenburg, Sweden. Patients living in the Västra Götaland County who were diagnosed with primary OSCC during the period 2005–2018 were included. The anatomic locations of the OSCCs corresponded to the following ICD-10 codes: C00.3, C00.4, C02.0, C02.1, C02.2, C03.0, C03.1, C04.0, C04.1, C04.8, C04.9, C05.0, C06.0, C06.1, and C06.2. The study was approved by the Swedish Ethical Review Authority (No. 2019-00790).

Data

Patients included were cross-checked against the medical records at all specialist clinics of Oral and Maxillofacial Surgery (OMFS) and Oral Medicine (OM) in Västra Götaland County. Information related to a preceding OPMD diagnosis and, if present, a strategy for monitoring, was extracted. All subtypes of OPMD diagnosis according to the latest WHO Expert Group Consensus report were included (23). OPMD diagnoses identified by searches in the medical records included OLs, erythroplakias, oral lichen planus and oral manifestations of Graft-versus-Host disease. In the next phase, the patients were divided into three groups (A–C; Table 1) according to whether or not there was a preceding OPMD diagnosis and whether or not the OPMD was monitored by a specialist in OM or OMFS. Follow-up was defined as the performance of at least one clinical control annually.

Table 1. *Descriptions of Groups A–C.*

Group A	Patients diagnosed with an OPMD and who underwent clinical follow-ups at regular intervals (<12 months) conducted by a specialist in OMFS or OM.
Group B	Patients diagnosed with OPMD but without a surveillance program at an OMFS or OM clinic. The majority of patients in Group B were sent back to the general dentist, with information given to both the patient and dentist regarding OPMDs, risk of MT, and recommendation for monitoring.
Group C	Patients with OSCC without any information in the OM or OMFS records regarding to a preexisting OPMD.

Information regarding gender, age, and lesion localization was additionally obtained from the medical records. Localization of the lesion was assigned based on the Union for International Cancer Control (UICC; 8th edition) (2) as: *floor of the mouth, hard palate, maxillary gingiva, mandibular gingiva, tongue* (anterior 2/3), and *Buccal mucosa.* Data regarding TNM classification and survival were obtained through cross-checking the included patients against the Regional Cancer Register of the western Sweden healthcare region. A study flow-chart is presented in **Paper I**.

Statistical analyses

To compare the groups regarding age, gender, stage, localization, T-stage, N-stage and M-stage, a pairwise test was used. For comparing age, the Student's *t*-test was used, and for disease stage and gender a Chi-square test was used. Fisher's exact test was used to compare localizations, T-, N- and M-stage.
To estimate the net survival in a relative survival setting the Pohar Perme method was used. To estimate the net survival, the death rate for the Swedish population was used. A log-rank type test, with pairwise groups, was used to analyze differences in net survival (112). To assess the influences of gender, age, group, localization and stage on overall survival, a Cox proportional hazards regression was used. Patients who were missing a clinical stage were excluded from the Cox model, as were patients with OPMDs of the *Hard palate*, due to their limited number. A Kaplan-Meier curve was used to estimate the overall survival. Five years after OSCC diagnosis, the follow-up was truncated.

To evaluate the assumption of proportional hazards, scaled Schoenfeld residuals over time were used. The only covariate for which a violation of the proportionality assumption was detected was the clinical stage. However, we considered that the regression could still be used, although the hazard ratio (HR) for stage should be interpreted with caution as an average effect over time.

A p-value <0.05 was considered as statistically significant in all analyses.

3.2 PAPER II

Patients

Patients with OL were identified through searches of the databases of four different specialist clinics (Departments of Oral and Maxillofacial Surgery and Oral Medicine and Pathology, Public Dental Health Service, at the Sahlgrenska University Hospital, Gothenburg, Region Västra Götaland and Department of Oral Medicine, Public Dental Health Service, Östra Hospital, at Sahlgrenska University Hospital, Gothenburg, Västra Götaland, Sweden; and Department of Oral and Maxillofacial Surgery at NU-hospital group, Trollhättan, Västra Götaland, Sweden). Patients with OL who were managed between January 1, 2003 and December 31, 2013 were candidates for inclusion. The aim was to have at least 5 years of follow-up for all patients.

The criteria for inclusion were: clinical OL diagnosis based on the WHO definition (26); histopathologically evaluated, reported with or without dysplasia; and access to clinical images of the lesion for re-evaluation. Patients with OSCC development within 6 months of the initial OL diagnosis were excluded, to reduce the risk of OSCC being present already at inclusion. All the patients with OL were cross-checked against the Swedish Cancer Register to attest the correct grouping of patients before division into the two groups of MT and non-MT. All of the patients who underwent MT developed OSCC within the site of the OL. The study was approved by the Regional Ethical Board Gothenburg, Sweden, (No. T613-17).

Data

Clinical subtype. OLs were divided as homogeneous or non-homogeneous, according to the WHO definition (26). The digital images of the lesions were assessed and referred into one of the two subgroups by three independent, blinded and experienced clinicians. If disagreement between observers occurred, the case was discussed to consensus. The intra- and inter-observed reliability levels were tested among the three observers by assessment of digital images of 30 OL cases. A re-evaluation was done four weeks later. The inter- and intra-rater kappa (κ)- coefficients were calculated by using Cohen's kappa test. The inter-rater κ-coefficient ranged from 0.7 to 0.83, and the intra-rater κ-coefficient ranged from 0.83 to 0.94. The inter-rater and intra-rater κ-coefficients were considered acceptable.

Histopathologic diagnosis. According to the classification extracted from the histopathologic reports, the OLs were divided into two subgroups, non-

dysplastic and dysplastic (regardless of dysplasia grade: mild, moderate or severe).

Anatomic localization. The OLs were categorized into six subgroups based on the affected anatomic location: inside lip (only intra-oral lesions were included), tongue, gingiva, floor of the mouth, hard palate, and buccal mucosa. Grouping of subsites was based on the clinical images and information obtained from the medical records. In the multivariable analysis, locations other than tongue were combined into a single group, which yielded the following comparison of anatomic subgroups: tongue vs other locations.

Lesion size was estimated based on the clinical images and information obtained from the medical records. OLs were then subdivided into a group with size ≥200 mm^2 and a group with size <200 mm^2, according to Holmstrup and coworkers (31).

Number of lesions. The patients were subdivided based on the number of lesions into either solitary or multiple OLs. The information on the number of lesions was obtained from the clinical images and medical records.

Smoking. The patients were assigned to smoker or non-smoker groups. If the patient had quit smoking more than 10 years before inclusion, he or she was considered to be a non-smoker.

Age. The ages of the patient resulted in a split into a dichotomous scale of <60 years and ≥60years, as reported Liu et al (32).

Statistical analyses

The primary outcome of this study was to evaluate the differences in MTR between homogeneous and non-homogeneous OL. For the analysis of the MTR in association with dysplasia: anatomic location, lesion size, number of lesions, age, tobacco smoking habits and gender were the secondary variables. To analyze the different factors associated with MTR, we used both univariable and multivariable Cox regression analyses. The multivariable analyses were used to investigate the internecine relationships between the studied factors. HRs were calculated with cause-specific Cox proportional hazards regression, to determine the biologic effects of the analyzed covariables in the presence of competing risks.

The follow-up time in the HR calculation was estimated from OL diagnosis to the occurrence of any of the following events: OSCC diagnosis, death, or last day of follow-up. "*Death due to other reasons*" was considered as a competing

event to the MT of OL (see Figure 2A in **Paper II)**. A cause-specific Cox regression analysis was performed taking the competing risks into consideration. The different factors studied were retained in the multivariable analysis if they were statistically significant. The Schoenfeld residuals was used to test *Assumptions of proportional hazards*. To calculate the cumulative incidence of OSCC, the *stcompet* macro devised by Enzo Coviello was used. The *stpepemori* macro was used to evaluate the equality of the cumulative incidence in the presence of competing risks (113). A *p*-value <0.05 was considered as statistically significant. The Stata/IC release 16.1 for Mac software (StataCorp LLC, College Station, TX, USA) was used for the statistical analyses.

3.3 PAPER III

Patients/samples

Patients who developed OSCC at the same anatomic site as a previously diagnosed OL were identified from the databases and medical records of the Departments of Oral and Maxillofacial Surgery and Oral Medicine, Public Dental Health Service, at the Sahlgrenska University Hospital, Gothenburg, Region Västra Götaland, Sweden. Formalin-fixed, paraffin-embedded (FFPE) specimens from the OLs and OSCC of each patient were obtained from the archives. As controls, FFPE specimens of patients with OL who had follow-ups conducted at one of the included departments without developing OSCC were used. The study was approved by the Regional Ethical Board Gothenburg, Sweden, (No. 739-10 and T613-17).

Tissue microarray construction

A tissue microarray (TMA) was constructed that included specimen from the OLs that developed into OSCC, the corresponding OSCC, and the OLs that did not develop into OSCC. The TMA was constructed in collaboration with the Tissue Microarray Center at Lund University, Sweden.

TMAs are paraffin blocks containing core biopsies of several different tissue samples (donors) that are re-embedded in a new recipient TMA block. The advantage of TMAs is that they allow simultaneous analysis of a large number of tissue samples.

Prior to the cores being acquired from the donor blocks, representative tissue areas of interest were identified and marked on hematoxylin and eosin (H&E)-stained slides. Cores of 1 mm from the donor blocks were placed in the recipient block using a semi-automated arraying machine. The cores were orientated in an X–Y axis system with an associated TMA key/map. We constructed two TMA blocks (A and B) for the included patients. In block A, two cores were placed from each specimen, while the remaining cores were placed in block B (0–2 from each included specimen). Only sections from block A were used in the present investigation.

Selection of genes and Fluorescence in situ *hybridization*

Genes frequently affected by CNAs in HNSCC were identified from the cBioPortal database. Based on detailed molecular analyses of 517 HNSCCs in

the database, we selected one frequently lost tumor suppressor gene encoding a kinase inhibitor and three frequently amplified/gained oncogenes. The latter encode a growth factor receptor, a transcription factor, and a cell cycle regulator. Copy number profiling of the selected genes were performed using fluorescence *in situ* hybridization (FISH).

FISH uses fluorescence-labeled DNA probes that are complementary to the specific gene locus studied. In addition to the gene-specific probes, all probes contained fluorescence-labeled, chromosome-specific centromere probes. The gene-specific probe and the centromere probe were labeled with different fluorophores (dual-color FISH). Using fluorescence microscopy, the probe signals could be detected in the specimens and, thereby also gains and losses of the respective genes.

The TMA blocks were cut in 4-µm-thick sections in a microtome. The sections were deparaffinized and treated with heat pretreatment solution citric, followed by protease treatment. The probes were subsequently added to the slides, followed by DNA denaturation at 80°C for 10 min. The hybridization was carried out at 37°C for 20–40 h. The sections were thereafter washed and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were analyzed in a fluorescence microscope equipped with an appropriate filter set. Images were produced using a digital FISH imaging system.

Statistical analyses

Differences in CNAs between the groups were analyzed with Fisher's exact test using the SPSS Statistics for Macintosh ver. 26.0 software package (IBM Corp., Armonk, NY). A difference giving a p-value <0.05 was considered to be statistically significant.

Further information on the inclusion process, patients' characteristics, TMA construction, and FISH analysis is available in **Paper III**.

3.4 PAPER IV

Patients/samples

FFPE specimens from patients with OSCC (N = 15) and patients with clinically and histologically healthy oral mucosa (N = 12) were acquired from the archives of the department of Oral Medicine and Pathology, University of Gothenburg, Sweden, and the department of Oral and Maxillofacial Surgery, Uppsala University, Sweden. The study was approved by the Regional Ethical Board Gothenburg, Sweden, (No. T427-13).

Sample preparation and immunohistochemistry

Sections (4 μm in thickness) were deparaffinized and incubated with antigen retrieval solution at 60°C overnight (Diva Decloaker; Biocare Medical, Concord, CA, USA), followed by incubation with the primary antibody for 30 min. The sections were then incubated with Envision horseradish peroxidase (HRP)-labeled polymer (DakoCytomation A/S, Glostrup, Denmark) for 30 min. To detect positively stained cells, 3,3′-diaminobenzidine (DAB) substrate (DakoCytomation) was added, together with hematoxylin for counterstaining. Sections without the addition of the primary antibody served as a negative control; no positive control was used.

The primary antibodies used were directed against:

- TET2 (ab127416, 1:100; Abcam, Cambridge, UK),
- 5mC (clone 33D3, 39649; 1:50; Active Motif, Carlsbad, CA, USA),
- 5hmC (39769; 1:500; Active Motif).

Histological analysis

Three areas were selected in each specimen for quantification. In the healthy oral epithelium, one randomly selected area close to the resection margin at each side of the specimen and one central area were randomly selected. In the OSCC specimens, three areas were randomly selected for quantification. The selected areas were in the size range of 50,000–240,000 μm^2. Images acquired using a light microscope (Leitz Wetzler; Leica Microsystems, Wetzlar, Germany) equipped with a Leica DC100 camera (Leica Microsystems) served as the source for cell counting. Positively stained cells were counted using the CellSense computer software (Olympus, Hamburg, Germany) at 100× magnification.

Statistical analyses

Differences in the median values and the ranges of 5mC, TET2 and 5hmC levels between the groups were analyzed with the Mann-Whitney *U*-test (SPSS Inc., Chicago, IL, USA). A difference giving a p-value <0.05 was considered to be statistically significant.

4 RESULTS

4.1 PAPER I

Patients, characteristics and groups

Totally, 739 patients with OSCC were included in the study. The characteristics of the patients are shown in Table 2. A pre-existing OPMD was identified in 162 patients. Ninety-four of the 162 patients with OPMD had been monitored with regular clinical follow-ups (Group A), whereas the remaining 68 OPMD patients had not received follow-up (Group B). OPMD diagnoses and follow-up are described in Table 3. Overall, 577 patients with OSCC did not have any previous diagnosis of OPMD at any of the OM or OMFS clinics participating in the study (Group C).

Survival rates and disease stages

The overall 5-year survival rate for the 739 patients was 54.1% (95% CI 50.5–57.9%) and the net 5-year survival rate was 61.5% (95% CI 57.2–66.1%). In Group A, patients with OPMD with follow-up had a significantly higher net survival rate, as well as lower T-classification, N-classification, and clinical stage compared to Group B ($p = 0.022$, $p < 0.001$, $p = 0.006$, $p < 0.001$, respectively) and Group C ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively) (Table 2, Figure 6). There were no statistically significant differences between Group B (patients with OPMD without follow-up) and Group C (patients with OSCC without previously diagnosed OPMD) with regards to net survival, T-classification, N-classification or clinical stage ($p = 0.143$, $p = 0.703$, $p = 1.000$, $p = 0.475$, respectively). The 5-year net survival rate for Group A was 90.0% (95% CI 80.3–100.8%), as compared to 68.3% (95% CI 54.5–85.7%) for Group B and 56.1% (95% CI 51.4–61.3%) for Group C (Figure 6). For stage I compared to the other stages of disease (II–IV), Group A compared to Group C and low age compared to high age were associated with significantly lower risks of death in the multivariable Cox regression analysis (Table 4). Patients with stage II disease had a HR of 1.5 ($p = 0.031$), stage III had a HR of 2.8 ($p < 0.001$), and stage IV had a HR of 4.5 ($p < 0.001$), as compared to stage I with respect to risk of dying. Patients in Group C showed a HR of 2.12 compared to Group A ($p = 0.002$) with respect to risk of dying. In the univariable analysis, there were statistically significant differences between Group C compared to both Group A and Group B, respectively, regarding gender and different anatomic localizations. However, in the multivariable Cox regression analysis, gender and anatomic localization did not significantly affect survival.

Table 2. *Demographic and clinicopathological characteristics of Group A, B and C. Reprinted from Jäwert F et al. Regular clinical follow-up of oral potentially malignant disorders results in improved survival for patients who develop oral cancer. Oral Oncol 2021;121:105469.*

		A	B	C	*p* (A/B)	*p* (A/C)	*p* (B/C)
N (%)		94 (12.7)	68 (9.2)	577 (78.1)			
Gender	Female	51 (54.3)	38 (55.9)	245 (42.5)	0.837	0.033	0.035
	Male	43 (45.7)	30 (44.1)	332 (57.5)			
Age; median (IQR)		69.0 [61.0, 77.0]	70.0 [63.0, 79.2]	68.0 [59.0, 78.0]			
Age; mean (SD)		67.4 (13.6)	70.2 (12.5)	67.8 (13.8)	0.176	0.780	0.169
Localization	Buccal mucosa	18 (19.1)	11 (16.2)	50 (8.7)	0.338	0.025	0.006
	Floor of the mouth	12 (12.8)	3 (4.4)	102 (17.7)			
	Hard palate	1 (1.1)	0 (0.0)	9 (1.6)			
	Mandibular gingiva	14 (14.9)	16 (23.5)	96 (16.6)			
	Maxillary gingiva	11 (11.7)	8 (11.8)	40 (6.9)			
	Tongue	38 (40.4)	30 (44.1)	280 (48.5)			
T-category	T1	51 (54.3)	18 (26.5)	164 (28.4)	<0.001	<0.001	0.703
	T2	29 (30.9)	21 (30.9)	185 (32.1)			
	T3	2 (2.1)	7 (10.3)	38 (6.6)			
	T4	11 (11.7)	22 (32.4)	189 (32.8)			
	T missing	1 (1.1)	0 (0.0)	1 (0.2)			
N-category	N0	84 (89.4)	48 (70.6)	401 (69.5)	0.006	<0.001	1.000
	N1	3 (3.2)	8 (11.8)	69 (12.0)			
	N2	6 (6.4)	12 (17.6)	98 (17.0)			
	N3	0 (0.0)	0 (0.0)	3 (0.5)			
	N missing	1 (1.1)	0 (0.0)	6 (1.0)			
M-category	M0	94 (100.0)	68 (100.0)	557 (96.5)	1.000	0.601	1.000
	M1	0 (0.0)	0 (0.0)	6 (1.0)			
	M missing	0 (0.0)	0 (0.0)	14 (2.4)			
Stage	Stage I	49 (52.1)	15 (22.1)	153 (26.5)	<0.001	<0.001	0.475
	Stage II	24 (25.5)	18 (26.5)	146 (25.3)			
	Stage III	4 (4.3)	10 (14.7)	52 (9.0)			
	Stage IV	15 (16.0)	25 (36.8)	216 (37.4)			
	Stage missing	2 (2.1)	0 (0.0)	10 (1.7)			

Table 3. *OPMD diagnosis of Group A and B. Follow-up intervals of Group A. Oral lichen planus (OLP), oral manifestation of Graft-versus-Host disease (GVH).*

		Group A	**Group B**
N (%)		94	68
OPMD	OL	71 (76)	53 (78)
	OLP	16 (17)	13 (19)
	Erythroplakia	3 (3)	2 (3)
	GVH	4 (4)	-
Follow-up interval	≤ 3 months	48 (51)	-
	≤ 6 months	33 (35)	-
	≤ 12 months	13 (14)	-

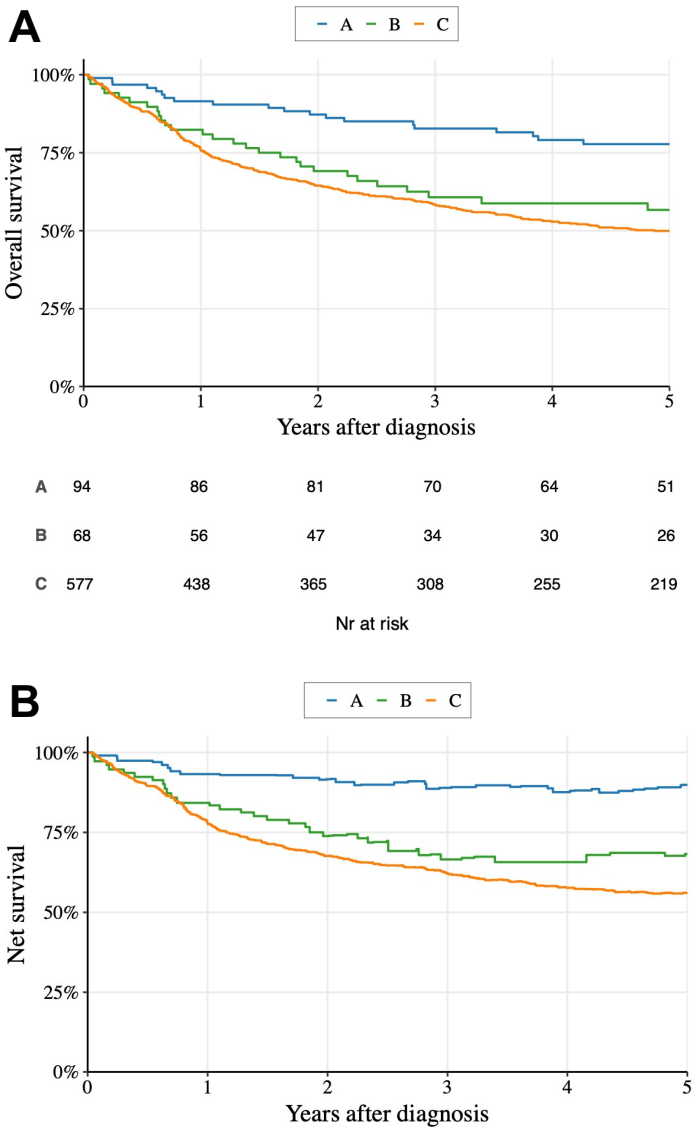

Figure 6. *Survival curves for Group A, B and C including number-at-risk. (A) overall survival and (B) net survival. Reprinted from Jäwert F et al. Regular clinical follow-up of oral potentially malignant disorders results in improved survival for patients who develop oral cancer. Oral Oncol 2021;121:105469.*

Table 4. *Multivariable Cox regression analysis showing hazard ratios (HR) in risk of death for included covariables. Reprinted from Jäwert F et al. Regular clinical follow-up of oral potentially malignant disorders results in improved survival for patients who develop oral cancer. Oral Oncol 2021;121:105469.*

		HR (95 % CI)	**p-value**
Gender	Male (N=393)	Ref.	
	Female (N=325)	1.03 (0.82 – 1.3)	0.785
Age (per 10 years)	(N=718)	1.70 (1.54 – 1.9)	<0.001
Group	A (N=91)	Ref.	
	B (N=68)	1.44 (0.80 – 2.6)	0.229
	C (N=559)	2.12 (1.32 – 3.4)	0.002
Localization	Tongue (N=344)	Ref.	
	Buccal mucosa (N=78)	1.01 (0.69 – 1.5)	0.941
	Floor of the mouth (N=114)	1.04 (0.74 – 1.4)	0.834
	Mandibular gingiva (N=126)	0.75 (0.55 – 1.0)	0.068
	Maxillary gingiva (N=56)	0.74 (0.48 – 1.1)	0.154
Stage	Stage I (N=215)	Ref.	
	Stage II (N=186)	1.52 (1.04 – 2.2)	0.031
	Stage III (N=66)	2.84 (1.81 – 4.4)	<0.001
	Stage IV (N=251)	4.53 (3.21 – 6.4)	<0.001

4.2 PAPER II

Patients and malignant transformation rate

In total, 234 patients diagnosed with OL were included in the study. OSCC development during the study period was observed for 27 patients (11.5%). The median follow-up time from initial OL examination to OSCC development was 49 months (range, 6–134 months). The follow-up time among patients who did not develop OSCC was 114 months (range, 3–234 months). Death was the first event for 50 patients, 11 of whom died before 5 years of follow-up. The median follow-up time of these 11 patients was 36 months (range, 3–59 months).

Clinicopathologic factors

In the multivariable analysis, non-homogeneous OLs displayed a 15.2-fold (range,4.5–52.0-fold) higher MTR compared to the homogeneous OLs ($p < 0.001$) (Table 5). Dysplastic OLs showed a 2.4-fold (range, 1.0–5.7-fold) higher MTR compared to non-dysplastic OLs ($p < 0.048$), while OLs located at the tongue had a 2.8-fold (range, 1.2–6.7-fold) higher MTR than OLs at other locations ($p < 0.018$). Twenty-four out of 74 (32.4%) cases if non-homogeneous OL, as compared to 3 out of 160 (1.9%) homogeneous OLs, transformed into cancer. Thirteen out of 30 (43.3%) dysplastic OLs underwent MT, as compared with 14 out of 204 (6.9%) non-dysplastic OLs. Overall, 16 out of 65 (24.6%) OLs located at the tongue transformed into OSCC in comparison to 3 out of 24 (12.5%) OLs located in the floor of the mouth, 3 out of 33 (9.1%) located in the buccal mucosa, 1 out of 14 (7.1%) located at the inside of the lip, 4 out of 48 (4.8%) gingival OLs, and none of the palatal OLs. The univariable analysis revealed that OLs with area >200 mm^2 had an increased MTR (Table 5). However, in the multivariable analysis, lesion size was not a significant factor.

Table 5. *Clinicopathological factors, rate of malignant transformation, follow-up in person-years and the uni- and multivariable Cox regression analyses. Reprinted from Jäwert F et al. Clinicopathologic factors associated with malignant transformation of oral leukoplakias: a retrospective cohort study. Int J Oral Maxillofac Surg. 2021;50(11):1422-1428.*

					Univariable Cox regression		Multivariable Cox regression	
Variable	Number of patients	Number of OSCC	Person-years	Rate per 1,000 person-years (95% CI)	Hazard Ratio (95% CI)	p-value	Hazard Ratio (95% CI)	p-value
Age at OL diagnosis (years)								
<60	102	12	1,006.8	11.9 (6.8–21.0)	Ref.			
≥60	132	15	1,160.3	12.9 (7.8–21.4)	1.05 (0.49–2.24)	0.90		
Gender								
Male	122	17	1,133.0	15.0 (9.3–24.1)	Ref.			
Female	112	10	1,034.1	9.7 (5.2–18.0)	0.64 (0.29–1.40)	0.26		
Clinical diagnosis								
Homogeneous OL	160	3	1,549.1	1.9 (0.6–6.0)	Ref.		Ref.	
Non-homogeneous OL	74	24	618.0	38.8 (26.0–57.9)	19.8 (5.9–65.7)	<0.001	15.2 (4.45–52.0)	<0.001
Uni-/multi-focal lesions								
Uni-	176	20	1,614.2	12.4 (8.0–19.2)	Ref.			
Multi-	58	7	552.9	12.7 (6.0–26.6)	1.02 (0.43–2.41)	0.97		
Smoker								
No	131	15	1,196.5	12.5 (7.6–20.8)	Ref.			
Yes	82	9	737.7	12.2 (6.4–23.4)	0.96 (0.42–2.19)	0.92		
Lesion size								
<2 cm²	119	7	1,139.0	6.1 (2.9–12.9)	Ref.			
≥2 cm²	115	20	1,018.7	19.6 (12.7–30.4)	3.14 (1.33–7.43)	0.009		
Location of lesion								
Tongue	65	16	553.9	28.9 (17.7–47.2)	Ref.		2.82 (1.19-6.68)	0.018
Gingiva	83	4	821.1	4.9 (1.8–13.0)	0.17 (0.06–0.52)	0.002		
Lip	14	1	146.7	6.8 (1.0–48.4)	0.24 (0.03–1.81)	0.17		
Floor of mouth	24	3	220.4	13.6 (4.4–42.2)	0.46 (0.13–1.59)	0.22	Ref.	
Buccal	33	3	298.5	10.0 (3.2–31.2)	0.34 (0.10–1.17)	0.087		
Palate	15	0	126.6	0				
Histopathology								
Non-dysplasia	204	14	1921.9	7.3 (4.3–12.3)	Ref.		Ref.	
Dysplasia	30	13	245.2	53.0 (30.8–91.3)	7.32 (3.44–15.6)	<0.001	2.39 (1.01–5.68)	0.048

4.3 PAPER III

CNAs in OLs and OSCC

CNAs were identified in both OLs and OSCCs for the selected genes. Approximately half of the analyzable OLs showed CNAs in at least one of the genes investigated, as compared to approximately three quarters of the OSCCs. CNAs were identified in OLs that subsequently transformed into OSCCs, as well as in OLs that did not undergo MT. CNAs were observed somewhat more frequently in OLs progressing to OSCC compared to OLs that did not, although the difference was not statistically significant. For further results, see **Paper III**.

4.4 PAPER IV

5mC, 5hmC and TET2 in OSCC

The immunohistochemical analysis of 5mC showed an increased number of positively stained cells per mm^2 in cases of OSCC compared to the cases with healthy oral epithelium ($p < 0.05$) (Figure 7). The range of numbers of 5mC-positive cells in the OSCC specimens was substantial. The healthy oral epithelium showed clear and distinct staining of 5hmC (Figure 8). The OSCC showed a significant reduction, almost a loss, of 5hmC ($p < 0.001$). The immunohistochemical staining pattern for TET2 was comparable with that seen for 5hmC, with significantly lower number of positively stained cells per mm^2 in cases of OSCC than in the healthy oral epithelium ($p < 0.001$). However, the staining intensity and pattern were not as strong as those seen with the 5hmC staining (Figure 8).

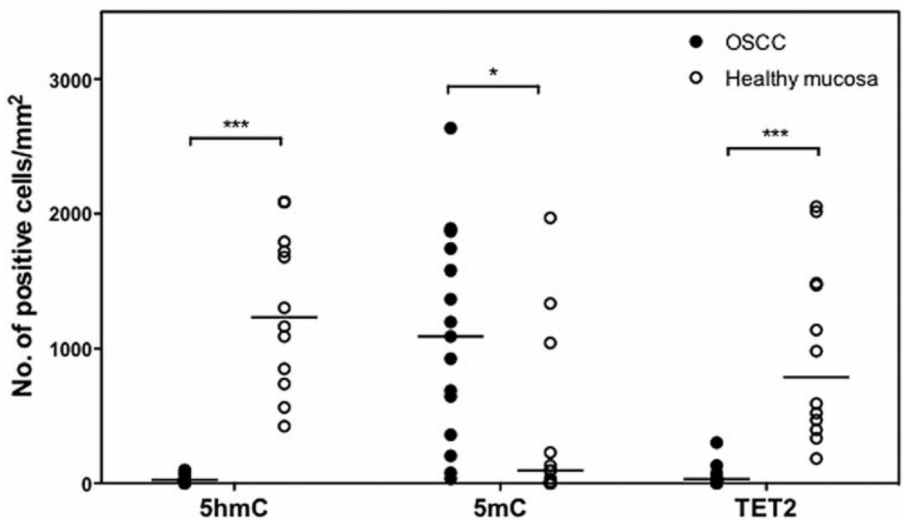

Figure 7. *Number and median values of positively stained cells / mm^2 for 5hmC, 5mC and TET2 in OSCC and healthy oral epithelium. * ($p < 0.05$), *** ($p < 0.001$). Reprinted with permission from Anticancer Research. Jäwert F, Hasséus B, Kjeller G, Magnusson B, Sand L, Larsson L. Loss of 5-hydroxymethylcytosine and TET2 in oral squamous cell carcinoma. Anticancer Res. 2013 Oct;33(10):4325-8. PMID: 24122999.*

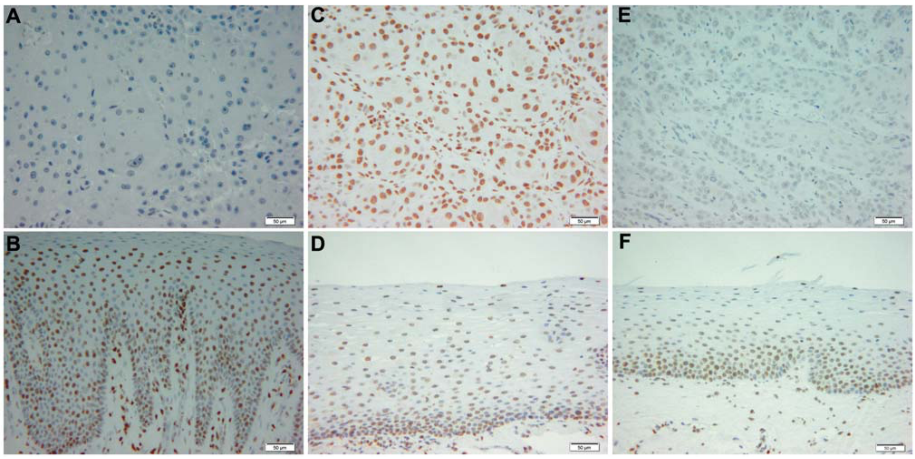

Figure 8. *Pictures from immunohistochemical analysis at 100 times magnification. 5hmC in (A) OSCC and (B) healthy oral mucosa. 5mC in (C) OSCC and (D) healthy mucosa. TET2 in (E) OSCC and (F) mucosa. Positively stained cells in brown. Printed with permission from Anticancer Research. Jäwert F, Hasséus B, Kjeller G, Magnusson B, Sand L, Larsson L. Loss of 5-hydroxymethylcytosine and TET2 in oral squamous cell carcinoma. Anticancer Res. 2013 Oct;33(10):4325-8. PMID: 24122999.*

5 DISCUSSION

The detection and treatment of OSCC before the tumor invades deep tissues and/or metastasizes are crucial for reducing morbidity and improving survival for these patients. Since many OSCCs are preceded by OPMDs, there is potential for early detection if MT occurs. Some cases may even be prevented. **Paper I** showed that almost 80% of the OSCCs that originate from OPMDs in the population of western Sweden are OLs. The optimal care and management strategies for patients with OL and the identification of patients who are at high risk for OSCC development are major clinical issues. This thesis describes the clinicopathologic factors and identifies new potential genomic – and epigenomic factors that are important for the prediction of MT. In addition, we demonstrate the benefits of follow-up programs for patients with OPMD.

Paper I revealed that patients with OPMD with follow-up, in the OM or OMFS setting, were diagnosed at a less-advanced clinical stage and had high survival rates if MT occurred. In contrast, patients with OPMD who had just received a diagnosis and information from an OM- or OMFS-specialist had a more advanced clinical stage at OSCC diagnosis and had a considerably lower survival rate. Surprisingly, there were no statistically significant differences regarding T- and N-classification or stage between the patients with OPMD without clinical follow-up and the patients with OSCC without known pre-existing OPMD. This suggests that OPMD diagnosis and information alone, in the absence of clinical follow-up, is not sufficient as an intervention for early OSCC detection. In our cohort, regarding the lead time to OSCC diagnosis, patients with OPMD without follow-up were comparable to patients with OSCC without a pre-existing OPMD diagnosis. This result was, however, based on a retrospective study with certain limitations. We hypothesized that patients with OPMD who were attending monitoring programs would be diagnosed at an early clinical stage if MT occurred and, thus, would have a high survival rate. However, as discussed in **Paper I**, the outcome was likely affected by additional factors, which we were not able to consider in this retrospective setup. Socioeconomic factors and comorbidities could affect the results, and knowledge regarding these topics was not available in the present study. The multivariable analysis showed that patients with OSCC in the group without pre-existing OPMD had twice as high risk of dying as patients with OPMD with follow-up, despite *clinical stage* being included in the analysis. This indicates that non-studied factors affect the outcome, which means that the different groups are not fully comparable. Patients with OPMD who attend a surveillance program may have a greater interest in their own health, resulting in a healthier lifestyle and fewer comorbidities compared to the other

groups of patients. However, there were no significant differences between the patients with OPMDs with follow-up and the patients with OPMDs without follow-up in the multivariable analysis when *clinical stage* was included. We consider that the main factor determinant of better survival in cases of OPMDs with follow-up is OSCC detection at a less-advanced clinical stage. This accords with the results of Ho and coworkers, who reported that the successful identification of OPMDs in oral mucosal screening programs in Taiwan was related to early OSCC diagnosis (114). In addition, the Society of Oral Medicine, Chinese Stomatological Association recently published an evidence-based position paper on how to manage OLs. It concluded that patients with OL should be monitored by an in-the-field, experienced clinician for early detection of MT (115). Despite the limitations of the present study, we consider the two OPMD groups (follow-up vs. non-follow-up), to be sufficiently comparable to conclude that patients with OPMD with follow-up are diagnosed at a less-advanced clinical stage and have a higher survival rate.

In the highly cited paper by van deer Waal (2009) (58), OPMD management concepts were discussed and strategies were recommended. A more or less lifelong follow-up program for OLs was recommended. Even if the median time to MT in our **Paper II** was 48 months, MT occurred at ≥11 years after initial OL diagnosis. These findings are in line with the lifelong follow-up recommendations made by van deer Waal (58). In that paper, van der Waal recommend a follow-up interval of 3–6 months. In **Paper I**, 86% of the patients with OPMD with follow-up had a monitoring regimen of at least once every 6 months. In addition, most of them were examined at least every third month. The remaining 14% of the patients were checked at an interval of 6–12 months. The latter subgroup of patients showed a lower survival rate when compared to patients with follow-up intervals ≤6 months (unpublished results). However, this statistically underpowered group also had a median age that was almost 10 years older than the patients with shorter follow-up intervals. The groups were, therefore, not comparable. However, we did not see any benefits associated with the follow-up interval of 6–12 months, and consider that our results are in agreement with the van der Waals recommendation of a monitoring interval of at least 3 to at least 6 months depending on risk factors discussed below.

Paper I, which evaluated the follow-up of OPMDs, did not include health economic aspects, even though the monitoring of patients with OL is expensive and resource-demanding for the healthcare system. However, as the multimodal therapy required for late-stage OSCC is expensive, it seems plausible that monitoring programs for patients with OL will be more cost-effective. If not, the increased survival and lower morbidity rates should be sufficient motivation for such monitoring to be put in place. The OPMD

diagnosis covers a heterogeneous group of lesions and conditions regarding both prevalence and MTR. The follow-up interval should be adapted taking into account the MTR linked to the specific diagnosis and the clinicopathologic risk factors for the specific case. Erythroplakias and high-risk OLs should be monitored more frequently. Oral lichen planus has, in general, a low MTR, so the value of monitoring strategies is less-obvious from the cost-benefit perspective (116, 117). Nevertheless, if monitored, the patient will probably be diagnosed at an earlier stage and have a better prognosis. To summarize, risk-stratification based on current knowledge about the MTRs in different OPMD subgroups can facilitate the design of clinically effective follow-up programs.

According to the van deer Waal management concept (2009) (58), patients with dysplastic OL should be seen once every third month, while patients with non-dysplastic OL should be seen once every sixth month. In addition to dysplastic OLs, we suggest, based on the results presented in **Paper II** and previous studies, that non-homogeneous OLs and OLs located at the tongue should also be considered as high-risk lesions, and therefore they should be monitored frequently. In our cohort, dysplastic OLs had a significantly high MTR (43%). However, a non-negligible fraction of the non-dysplastic OLs did undergo MT. Of the 27 OLs that underwent MT, as described in **Paper II**, 14 were non-dysplastic. This highlights the importance of taking other factors into consideration in predicting the risk for patients with OL, especially for non-dysplastic OL cases. In addition, the assessment and grading of dysplasia may be rather subjective, with significant inter- and intra-observatory variabilities affecting the assessment. Our results indicating a high MTR of non-homogeneous OLs are in line with the findings of Holmstrup et al (31) and Liu et al (32). In our material, < 2% (3/160) of the homogeneous OLs transformed into cancer, despite a median follow-up of 9.5 years. A similar low MTR for homogeneous OLs has been reported in a Danish cohort (31), with a reported MTR of 3% regarding both surgically treated and non-treated homogeneous OLs. In our material, the clinical sub-diagnosis was a strong predictive factor for MT. In the multivariable Cox regression analysis, non-homogeneous OLs had a HR of 15.2 for risk of undergoing MT compared to homogeneous OLs. Our observation of the tongue as a high-risk location is supported by the results of Liu et al (32), and Evren et al (35). In addition, large-sized OLs must also be considered for more-frequent monitoring, as groups have linked these large OLs to a high MTR (31, 36). For OLs that do not display these high-risk characteristics, a monitoring interval of 6 months appears to be appropriate.

The primary aim of **Paper II** was to investigate the clinical OL sub-diagnosis with respect to MTR. All the included OLs were ‘clinically’ re-evaluated and grouped into either homogeneous or non-homogeneous OLs by three

experienced, blinded clinicians who reviewed clinical photographs. This is considered as a strength of the present study. In addition, patients had a long follow-up, with a median time of 114 months for patients who did not develop OSCC. However, there were also some limitations. The histopathologic diagnosis of each and respective OL was not re-evaluated. In addition, the material was statistically underpowered for an optimal evaluation of the impacts of the different anatomic locations on MT, resulting in a questionable grouping in the multivariable analysis. Treatments aimed at preventing the MT of OLs are widely discussed in the literature. In **Paper II**, the type of treatment was not taken in account. This is also a limitation that may have affected the outcome of the study. So far, there have been no conclusive prospective and randomized controlled trials evaluating the effects of surgical removal of OLs to prevent MT. Retrospective studies have reported both an increased and a decreased risk of MT when OLs have been surgically treated (31, 51). However, there is a distinct benefit if the lesion is removed. Since, an excision enables histologic examination of the whole lesion. Studies have reported a high extent of unexpected carcinomas in completely excised OLs and erythroplakias where the incisional biopsy did not show OSCC. Holmstrup and coworkers reported that 7/101 (7 %) excised lesions that showed dysplasia at the incisional biopsy and/or were located at the tongue/floor of the mouth harbored carcinoma in the excisional specimen (57). Thomson and coworkers reported that 71/590 (12 %) of the lesions showing dysplasia at the incisional biopsy did reveal unexpected carcinoma when the lesion was completely removed (118). Gillvetti and coworkers reported that 19/120 (18 %) lesions displaying dysplasia at the incisional biopsy, contained unexpected carcinoma in the excisional specimen (51).

A correlation between positive excision margins and both recurrence rate and MTR have recently been reported (51). Inadequate excision margins and field cancerization were factors highlighted by Holmstrup and Dabelsteen (44) as affecting the success rate of surgically treated OLs. The ethical dilemma faced by the surgeon of performing mutilating surgery on a patient with a non-malignant diagnosis will probably result in non-adequate excision margins in many patients. However, to remove OLs with positive or narrow margins is also mutilating. In addition, the risk of recurrence is increased (51). The assessment and planning for adequate margins, including the subclinical circumscription of a lesion is a delicate issue. Tools that, for example, employ autofluorescence and narrow band imaging appear to be promising and superior to clinical visualization alone (119-123). Further and future prospective studies are though needed for these techniques. In addition, prospective randomized clinical trials evaluating the surgical treatment of OL with long follow-up is of outmost importance. Until such results are available,

an individual assessment of each specific case must be made, considering the benefits and disadvantages of the respective strategies. Again, follow-up of OLs is of the outmost importance, regardless of whether treatment or not, has been attempted.

To obtain further information about the risk prediction of OLs, information regarding the genomic status of a lesion may be of significant value. This information may also be useful for assessing the 'clinical healthy' tissue adjacent to the lesion. Many studies have aimed to evaluate biomarkers for predicting the risk of MT of OLs (95, 102). However, to date, no reliable biomarkers have become available. From the genomic perspective, OSCCs and OLs are heterogeneous. A number of different genes are involved in OSCC development, and none of the currently known drivers occur in all cases. In addition, we and others have detected known OSCC drivers also in non-progressing OLs. However, knowledge about the genomic status of a specific case may provide additional information, and can be added to clinicopathologic factors in predicting the risk of MT. For example, aneuploidy (deviation from the normal diploid chromosome number) has been reported to have a predictive value related to the MT of OPMDs (124). When aneuploidy is combined with dysplasia grading it adds additional information to the risk prediction of OPMDs (125, 126). In addition, and even more importantly, specific genomic alterations may be targets for new therapeutic strategies that employ a 'precision medicine' approach. Interestingly, in the Erlotinib Prevention of Oral Cancer trial, Williams and coworkers evaluated the ability of Erlotinib (an EGFR tyrosine kinase inhibitor) to reduce MT of OPMDs (105). Patients with OPMDs were randomized to the Erlotinib group (N = 75) or control group (N = 75). Even though Erlotinib was found not to affect the MTR in this study, this was an interesting study that represents a new treatment strategy to prevent MT.

In **Paper III**, we conducted copy number profiling of: four known HNSCC drivers in OLs that progressed to OSCCs; their corresponding OSCCs; and OLs that did not progress to cancer. CNAs in these genes were detected not only in the OSCCs, but also in the OLs. The CNAs were, not surprisingly, detected more frequently in OSCCs than in OLs. Approximately half of the OLs showed CNAs in at least one of the four genes studied. CNAs were detected in both OLs undergoing and not undergoing MT, although the CNAs of some genes were detected somewhat more often in OLs undergoing MT. An increased copy number of the epidermal growth factor locus has earlier been reported in OPMDs that transformed into OSCCs (103-105). Loss of heterozygosity in chromosome 9p (including *CDKN2A*) has previously been reported to be more frequent in progressing OPMDs (106, 107). The results of

our study, together with those of previous studies, indicate that certain CNAs are early genomic events in OSCC development in certain subsets of patients.

The patients included in the studies described in **Paper III** were well-characterized clinically and histopathologically. For example, patients in the OL group without transformation to OSCC had a median follow-up of 102 months (range, 50–268 months), conducted by an OM- or OMFS-specialist, which is a strength of the study. The non-MT group was matched with the MT group regarding gender, age, dysplasia grade, and lesion localization. The groups were not matched regarding clinical sub-diagnosis, i.e., homogeneous and non-homogeneous. In the group with OL transforming into OSCC, 13/14 were non-homogeneous, as compared to only 7/14 in the group that did not develop OSCC. The observation that few homogeneous OLs progress to OSCCs is in agreement with our findings in **Paper II**. The study includes relatively few patients and CNAs were only studied for four genes, which is a limitation of the study. To save valuable material and to facilitate the FISH analysis, we constructed a TMA of the included specimens. The restricted tissue amounts in the TMA cores (as opposed to analyses conducted on an entire specimen) is also a limitation. This issue may be analogous the limitations of incisional biopsies relative to complete surgical removal, as discussed above. Even though the representative nature of the TMA cores is assessed and selected based on histopathologic aspects, it is important to bear in mind the intratumor genomic heterogeneity reported for both OSCCs and OLs (127-129). To reduce the influence of this issue, we used two 1-mm cores instead of 0.6-mm cores from each case (130). There is also a risk that while some OLs in the non-MT group harbored CNAs that over time would result in OSCC development, they were successfully protected against future MT by the surgical excision.

In **Paper III**, we demonstrate that the CNAs of four known driver genes occur in both OSCCs and OLs. This indicates possible roles for the CNAs of some of these genes in the development and progression of subsets of OLs. For further discussion of this topic, see **Paper III**.

Paper IV compares the epigenetic factors 5mC, TET2 and 5hmC between OSCC and healthy oral epithelium. This was the first published study evaluating TET2 and 5hmC in OSCCs and healthy oral epithelium. Our results show differences in the numbers of immunohistochemically stained cells for 5hmC, TET2 and 5mC between OSCC and the healthy oral epithelium. The levels of 5hmC were significantly reduced in the OSCC. The expression of TET2 enzyme corresponded to the expression level seen for 5hmC, with decreased expression levels in OSCC. This indicates a possible role for the

TET2 enzyme in the loss of 5hmC seen in our OSCCs. A higher level of expression of 5mC in OSCC, as observed in our study, may be the result of accumulation of 5mC following the reduction in TET2 level and, as a consequence, non-oxidation of 5mC to 5hmC.

As mentioned above, reduced levels of 5hmC have been reported in different cancers. Our finding of low levels of 5hmC in OSCC has been confirmed by studies conducted by Wang et al (131) and Misawa et al (132). Surprisingly, and in contrast to what has been reported for other cancers, Wang and colleagues reported that OSCCs with high levels of 5hmC were associated with reduced survival. Low levels of 5hmC have previously been correlated with advanced disease and low survival rate (90, 93). Recently, 5hmC has also been investigated in OPMDs. Cuevas-Nunez and coworkers have reported decreased levels of 5hmC in ODs and OSCCs, as compared to oral fibromas, frictional keratosis and oral lichen planus specimens (133).

Whether the loss of 5hmC is a cause or a consequence of cancer has been discussed in the literature (134). In a study on melanoma, Lian et al. (90) showed that downregulation of TET2 is a mechanism for reducing the levels of 5hmC. In the same study, it was also shown that reintroducing the TET2 enzyme re-established the levels of 5hmC in melanoma cells *in vitro* and in less-aggressive tumors in an animal model. This result indicates functional roles for 5hmC and TET2 in melanoma. Loss of 5hmC has been suggested as a potential biomarker for melanoma, and the TET enzymes have been discussed in terms of their therapeutic potential.

Huang and coworkers (135) have reported reduced levels of TET2 in HNSCC compared to healthy tissues. This is in line with our findings for TET2 in OSCCs. It was also shown that low levels of TET2 correlate with advanced clinical stage and low survival rate. Furthermore, similar to what was shown by Lian et al. (90) for melanoma, restoration of TET2 in HNSCC cell lines resulted in reduced cell proliferation *in vitro* and in smaller tumors in an animal model (135).

Among the limitations of our study, there is no correlation between the results and the clinical characteristics. In addition, we only investigated the global level of DNA methylation. The numbers of 5mC-positive cells varied widely between the different OSCC specimens. 5mC could be involved in tumor development due to increased or reduced levels, depending on the type of gene affected. In other words, the global methylation level provides only limited information. Instead, 5mC should be studied on a gene-specific level and related to protein expression. Interestingly, hypermethylation of a well-known

tumor suppressor gene, that showed copy number loss in OSCCs and OLs in Paper III, has been reported in OSCCs and in ODs (136 – 139). In addition, hypermethylation of the gene was more commonly detected in ODs that progressed to OSCC than in ODs that did not progress to OSCC. Indicating multiple pathways towards loss of function of this tumor suppressor in the development of OSCCs.

To summarize, loss of 5hmC and TET2 in OSCC is an interesting topic. 5hmC and TET2 may be candidates for prognostic biomarkers, and may even be candidates for therapeutic targets. Studies are needed to investigate 5hmC and TET2 in OLs that are undergoing MT, in comparison to OLs that are not undergoing MT, and to elucidate further the functional roles of 5hmC and TET2 in OSCCs.

6 CONCLUSION

In this thesis, we demonstrate that follow-up programs for patients with oral leukoplakias result in early detection and higher survival rates, if cancer develops. As the monitoring of these patients is expensive and resource-demanding for healthcare systems, high-risk patients should be prioritized. We have identified clinical, histopathologic, genomic and epigenomic factors that have potential to predict which patients with OL are at high risk of cancer development. These factors could be useful for stratifying those patients who should be prioritized and scheduled for frequent clinical follow-ups.

Paper I

An absolute majority of oral potentially malignant disorders that progress to oral squamous cell carcinomas (OSCC) in the population of western Sweden are oral leukoplakias (OLs).

Regular clinical follow-ups of OLs are favorable for the patients, since they result in earlier detection of cancer and improved survival, if malignant transformation occurs.

Paper II

Non-homogeneous OLs have a significantly higher MTR compared to homogeneous OLs. Dysplastic OLs have a higher rate of malignant transformation compared to non-dysplastic lesions. In addition, OLs located at the tongue more frequently transform into malignant tumors, as compared to lesions located at other anatomic locations in the oral cavity. This should be taken into consideration in clinical decision-making related to the follow-up strategies for these patients. Finally, malignant transformation of OL may occur long after the primary diagnosis, indicating the importance of a lifelong follow-up strategy for high-risk OLs.

Paper III

Copy number alterations (CNAs) of known cancer-driving genes occur not only in OSCC, but also in OL. This suggests roles for CNAs of certain driver genes in the development and progression of subsets of OLs. CNAs may, therefore, be early genomic events in OSCC development in certain subsets of patients.

Paper IV

The levels of 5-hydroxymethylcytosine are significantly lower in OSCC than in healthy oral epithelium. The levels of ten-eleven-translocation-2 correspond to the reduced expression of 5-hydroxymethylcytosine in OSCC, indicating a possible role for the enzyme in the loss of 5-hydroxymethylcytosine seen in OSCC.

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APPENDIX

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