ORAL LEUKOPLAKIA, HUMAN PAPILLOMAVIRUS AND CANCER TRANSFORMATION

Factors related to human papillomavirus infection and cell proliferation

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Cover illustration: Leukoplakia (left) transforms into oral squamous cell carcinoma (right). Photo: Dr. Maria Westin.

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"Success is 1% inspiration, 98% perspiration, and 2% attention to detail"
Phil Dunphy

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ABSTRACT

Oral leukoplakia (OL) is clinically diagnosed as a white oral lesion that cannot be scraped of or diagnosed as any other type of oral lesion. OL has the potential to transform into oral squamous cell carcinoma (OSCC). The gold standard treatment is a combination of surgical excision if possible, and surveillance. Despite complete removal, the risk of cancer or recurrence remains high. A major clinical problem is to predict which patients that have an OL that turns into OSCC. Infection with human papillomavirus (HPV), especially high risk (HR) HPV types has been attributed a role in cancer transformation of OL. HPV is a ubiquitous virus transmissible between humans and with a global variation in prevalence and known to cause cancer of the cervix uteri but also an important factor in oropharyngeal cancer. The overall aim of this thesis was to investigate the influence of HPV infection in OL, which clinical-, histopathological and treatment factors that affect the recurrence rate after surgical removal and to investigate molecular markers connected to cell proliferation and cancer transformation.

The thesis is based on five scientific questions addressed in four different studies:
Study I*: Is there a correlation between overexpression of the tumour suppressor protein p16 and high-risk HPV infection in leukoplakia and OSCC?* In this study the level expression of p16 in OL was assessed by immunohistochemistry and in the same group of patients OL tissue samples were analysed by Real-Time polymerase chain reaction (RT-PCR) for presence of twelve high-risk and two low-risk HPV types. In parallel, p16-expressing OSCC were analysed for the same HPV types.

Study II*: Has the prevalence of high-risk HPV in leukoplakia changed over time? Does the prevalence differ between Sweden, Romania and Brazil?* In contemporary and historical patient cohorts from the three countries OL samples were analysed with RT-PCR for 12 high-risk and

two low-risk HPV types. In patients with cancer transformation of their OL tumour samples were also screened for presence of the same HPV types.

Study III: *Which clinical and anamnestic factors correlate with the recurrence of leukoplakia after surgical removal?* Patients with OL that were surgically removed comprised the study cohort. At inclusion anamnestic and clinical factors were registered together with results of the histopathological examination. Study subjects underwent follow-up visits at 3-6 months post-surgery according to the study protocol. Recurrence was defined as reappearance of OL at the primary lesion site.

Study IV: *Can expression of the cell proliferation biomarkers p53, p63, podoplanin and Ki-67 predict the recurrence of leukoplakia after surgical excision***?** In this study patients with recurring vs non-recurring OL were compared regarding molecules known to be part of or influence cycle regulation. Immunohistochemistry was utilized and cell quantification was performed on digitalised images.

The results showed that:

- A high expression level of the tumour suppressor protein p16 is not a stable biomarker for presence of high-risk HPV in OL or OSCC **(Study I)**.
- High-risk HPV were found in low levels in Brazilian OL patients but in none of the Swedish or Romanian OL patients. Nor was any difference in HPV prevalence registered when comparing historical and contemporary cohorts. OSCC preceded by OL were all high-risk HPV negative **(Study II)**.
- The cumulative OL recurrence incidence after surgical excision was 45% after 4 years and 49% after 5 years. Non-homogeneous OL and use of snuff were significantly correlated with recurrence after surgical removal. Recurrence was also significantly associated with OSCC development at the primary lesion site **(Study III)**.
- In exploring if the biomarkers p53, p63, Ki-67 and podoplanin could predict recurrence after surgical treatment, p63 overexpression was identified to have a significant correlation to recurrence **(Study IV)**.

In summary, this thesis adds new knowledge regarding OL and HPV infection, surgical treatment outcome, cancer transformation and potentially useful biomarkers. But the thesis also points out future research avenues. Other HPV types than what have been investigated in this thesis may disclose a causative correlation between HPV, OL and cancer transformation. To date, surgical treatment of OL shows high recurrence rates, which points for a need for new techniques in determining surgical margins. This could be possible with emerging new non-invasive *in vivo* diagnostic tools. The potential of using a combination of biomarkers connected to cell proliferation as predictors for recurrence but also cancerous transformation is a *modus operandi* that in the future could be implemented in creating treatment decision algorithms for OL.

Keywords: potentially malignant oral disorders, recurrence, virus

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SAMMANFATTNING PÅ SVENSKA

En vit fläck i den orala slemhinnan som inte kan skrapas bort eller diagnosticeras som någon annan typ av oral slemhinneförändring kallas för oral leukoplaki, en förändring som kan omvandlas till oral skivepitelcancer (OSCC). Ett stort kliniskt problem med dessa förändringar är att förutsäga vilka av dem som kommer genomgå malign transformation. Standardbehandlingen består av kirurgiskt avlägsnande som sedan följs av regelbundna kontroller av munslemhinnan. En fråga som har diskuteras under en tid är huruvida en infektion med humant papillomvirus (HPV) föregår den maligna transformationen av leukoplakier eller ej.

Avhandlingen baseras på fem vetenskapliga frågor som behandlats i fyra olika studier:
Studie I: *Finns det ett samband mellan överuttryck av tumörsuppressorproteinet p16 och en högrisk HPV-infektion i leukoplakier och OSCC?*
Studie II: *Har prevalensen av högrisk HPV i leukoplakier förändrats över tid? Skiljer sig HPV-prevalensen mellan tre geografiska regioner: Sverige, Rumänien och Brasilien?*
Studie III: *Vilka kliniska och anamnestiska faktorer korrelerar med recidiv av leukoplakier efter kirurgisk avlägsnande?*
Studie IV: *Kan uttrycket av biomarkörerna p53, p63, podoplanin (PDPN) och Ki-67 förutsäga ett recidiv efter kirurgisk excision av leukoplakin?*

Patienter och metoder: Klinisk information och biopsimaterial samlades in från patienter som ingår i en prospektiv, longitudinell multicenterstudie i Sverige och från patienter med leukoplakier som erhölls efter registersökning vid avdelningarna för oral medicin och patologi, Göteborgs universitet, Sverige, Sao Paulo, Brasilien och Bukarest, Rumänien. De huvudsakliga laboratorieteknikerna som användes var immunohistokemi som visualiserade p16-, p53-, p63-, podoplanin- och Ki-67-uttryckande celler och en realtids-PCR inriktad på 12 högrisk HPV-typer och två lågrisk HPV-typer.

Resultat: I *studie I* observerades överuttryck av p16 i 18% av de analyserade leukoplakierna men ingen av de undersökta HPV-typerna detekterades. I p16-positiva orala skivepitelcancrar innehöll 38% av dem DNA från HPV16. I *studie II* upptäcktes ingen av de undersökta HPV-typerna i de svenska och rumänska kohorterna medan det i de brasilianska kohorterna visade sig att 3% av leukoplakierna innehöll HPV-DNA från de analyserande HPV-typerna. Ingen ökning av HPV prevalensen hos leukoplakier påvisades över tid. De leukoplakier som genomgick en malign transformation innehöll inga av de undersökta HPV-typerna och lika så var den efterföljande cancern HPV-negativ. I *studie III* recidiverade 42% av leukoplakierna efter en kirurgisk excision. Femtiosex procent av den icke-homogena leukoplakin återkom och bland snusare återkom 73% av leukoplakierna. Icke-homogena leukoplakier och användning av snus visade en signifikant korrelation med recidiv efter kirurgisk excision ($p = 0,021$ respektive $p = 0,003$). Leukoplakier som återkom efter kirurgisk excision visade sig också var signifikant korrelerad med malign transformation ($p < 0,001$). I *studie IV* var uttrycket av

tumörsuppressorproteinet p63 signifikant högre i leukoplakier som recidiverade efter kirurgisk excision jämfört med leukoplakier som inte recidiverade (p = 0,047). Uttrycket av Ki-67, p53 och podoplanin visade ingen signifikant skillnad mellan recidiverande jämfört med icke-recidiverande leukoplakier (p = 0,085; p = 0,17; p = 0,25 respektive)

Slutsatser: *Studie I* - Överuttryck av p16 är inte en pålitlig biomarkör för detektion av en infektion med högrisk-HPV hos patienter med leukoplakier eller OSCC. *Studie II* - I Sverige, Brasilien och Rumänien är förekomsten av högrisk-HPV i leukoplakier låg och prevalensen har inte förändrats under den undersökta tidsperioden. Inte heller de skivepitelcancrar som efterföljde leukoplakin innehöll något högrisk-HPV. *Studie III* - De enda kliniska parametrar som kan förutsäga recidiv av leukoplakier är den icke-homogen typen och patienter som använder snus. Recidivering av en kirurgiskt avlägsnad leukoplaki är också en riskindikator för cancerutveckling. *Studie IV* - Överuttryck av p63 var signifikant associerat med recidiv av leukoplakin efter kirurgisk excision.

LIST OF PAPERS

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

I. **Sundberg J**, Korytowska M, Miranda Burggos P, Blomgren J, Blomstrand L, De Lara S, Sand L, Hirsch JM, Holmberg E, Giglio D, Öhman J, Kovács A, Horal P, Lindh M, Kjeller G, Hasséus B. Combined testing of p16 tumour-suppressor protein and human papillomavirus in patients with oral leukoplakia and oral squamous cell carcinoma. *Anticancer Research* 2019; 39: 1293-1300.

II. **Sundberg J,** Öhman J, Korytowska M, Wallström M, Kjeller G, Andersson M, Horal P, Lindh M, Giglio D, Kovács A, Sand L, Hirsch JM, Araújo LM, Mamana Fernandes de Souza AC, Parlatescu I, Dobre AM, Hinescu ME, Henrique Braz-Silva P, Tovaru S, Hasséus B. High-risk human papillomavirus in patients with oral Leukoplakia and oral squamous cell carcinoma — A multi-centre study in Sweden, Brazil and Romania. *Oral Diseases* 2020; 00:1–10. DOI: 10.1111/odi.13510

III. **Sundberg J,** Korytowska M, Holmberg E, Bratel J, Wallström M, Kjellström E, Blomgren J, Kovács A, Öhman J, Sand L, Hirsch JM, Giglio D, Kjeller G, Hasséus B. Recurrence rates after surgical removal of oral leukoplakia – A prospective longitudinal multi-centre study. *PloS ONE* 2019: 14(12): e0225682.

IV. **Sundberg, J**. Pandey Dhakai S, Giglio D, Holmberg E, Kjeller G, Kovács A, Tokozlu B, Öhman J, Sapkota D, Hasséus B. Expression of p53, p63, podoplanin and Ki67 in recurring vs. nonrecurring oral leukoplakia. *In manuscript* 2020.

CONTENT

ABBREVIATIONS

CI	Confidence Interval
CIS	Carcinoma In Situ
C_t	Cycle threshold
FFPE	Formalin-Fixed and Paraffin-Embedded
HNSCC	Head and Neck Squamous Cell Carcinoma
HR-HPV	High-risk Human Papillomavirus
HR	Hazard Ratio
IHC	Immunohistochemistry
ISH	In-Situ Hybridisation
LR-HPV	Low-risk Human Papillomavirus
OL	Oral Leukoplakia
OPSCC	Oropharyngeal Squamous Cell Carcinoma
ORF	Open Reading Frame
OSCC	Oral Squamous Cell Carcinoma
PAD	Pathological Anatomical Diagnosis
PCR	Polymerase Chain Reaction
PDPN	Podoplanin
PMOD	Potentially Malignant Oral Disorder

1 INTRODUCTION

1.1 ORAL LEUKOPLAKIA

Oral leukoplakia (OL) involves the diagnosis of a white lesion in the oral cavity that carries an increased risk of malignant transformation.

The WHO defines leukoplakia as a "*white plaques of questionable risk, (other) known diseases and disorders that carry no increased risk of cancers having been excluded*" (1). This means that leukoplakia is an exclusion diagnosis, in cases in which other diagnoses are ruled out. A tentative diagnosis is made when a white lesion is found in the oral mucosa. A definitive diagnosis is reached after all other aetiological causes have been excluded and the histopathological examination has not confirmed any other specific disorder (1).

Leukoplakia is most often an asymptomatic disorder and is usually first discovered by a general dental practitioner during a routine dental check-up.

Leukoplakia belongs to a group of oral disorders that have the potential to transform into oral squamous cell carcinoma (OSCC). This group of potentially malignant oral disorders (PMOD) was reviewed in a consensus conference in 2005, with the outcome being presented in a position paper (1). Other PMOD that belong to this group are erythroplakia, palatal lesions in reverse smokers, submucosal fibrosis, actinic keratosis, discoid lupus erythematosus, and oral lichen planus. All of these disorders have various levels of risk for malignant transformation

1.1.1 CLINICAL ASPECTS

There are two main clinical subgroups of leukoplakia, homogeneous and non-homogeneous (Figure 1). The diagnosis is based on the clinical features where the homogeneous leukoplakia is characterised as a "*uniformly flat, thin and exhibit shallow cracks of the surface keratin*" (1). The non-homogeneous leukoplakia has more-diverse clinical features, in that a mixture of hyperkeratotic, erythematous, speckled, nodular and verrucous areas may be seen (1).

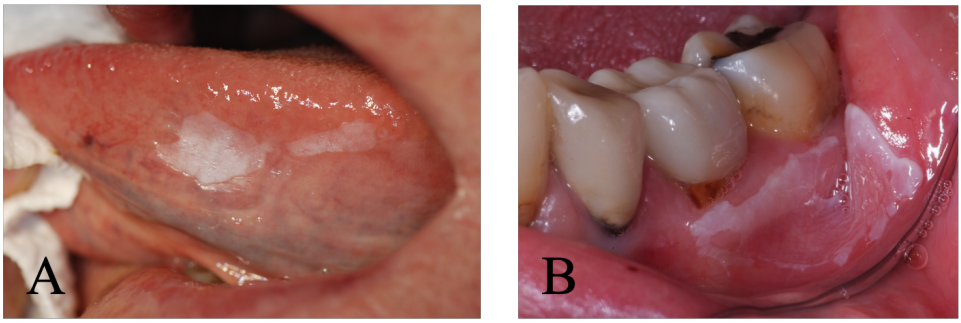

Figure 1. A) Homogenous leukoplakia on the lateral border of the tongue and B) non- homogeneous leukoplakia on the gingiva and buccal mucosa.

1.1.2 HISTOPATHOLOGICAL ASPECTS

Leukoplakia is a clinical diagnosis and is not used as a histopathological diagnosis. The histopathological examination is an important complement to the clinical diagnosis and a crucial part in the risk assessment of the leukoplakia, depending on the presence of dysplasia or not. The pathological anatomical diagnosis (PAD) is based on the histopathological features of the tissue. Since leukoplakia is a white patch in oral mucosa, one of the main histopathological findings is keratosis or hyperkeratosis in the superficial part of the epithelium, the stratum corneum. One important aspect of the histopathological examination is to check for the presence of epithelial dysplasia, as this is the gold standard for the diagnostic pathology. The histopathological diagnosis relies on the absence or presence of epithelial dysplasia. The evaluation of dysplasia severity is based on observations of the architecture of the epithelium and cellular atypia. The microscopic features of leukoplakia include: basal cell hyperplasia, loss of polarity of basal cells, increased number of and abnormalities of mitotic figures, irregular epithelial stratification, loss of epithelial cell cohesion, and drop-shaped and keratin pearls within the rete ridges. The grades of dysplasia are: mild, moderate, and severe (Figure 2). Mild dysplasia is characterised by cellular abnormalities and atypia extending to the lower one-third of the epithelium. Moderate dysplasia is when atypical cellular changes extend to the middle third of the epithelium. Severe dysplasia is when the cellular abnormalities extend over the middle-third of the epithelium, but not all the way up. When the cellular abnormalities extend all the way to the top of the epithelium it is referred as carcinoma in situ (CIS) (2, 3).

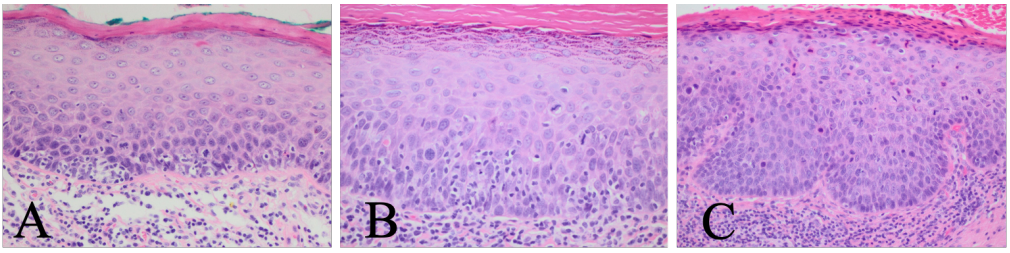

Figure 2. Oral epithelial dysplasia. A) Mild dysplasia. B) Moderate dysplasia. C) Severe dysplasia

The WHO grading system is widely applied in clinical and pathological routines. However, the three-part scale is debated, as the grading system has certain limitations. The main limitation is the high intra- and inter-variability among different pathologists, leading to poor reproducibility of the grading system (3). To overcome these deficiencies, a binary grading system has been suggested that uses the same criteria as the WHO grading system but that instead uses the categories of "low-grade" dysplasia and "high-grade" dysplasia (4).

1.1.3 PREVALENCE OF LEUKOPLAKIA

Leukoplakia is one of the most frequently detected PMOD in the oral mucosa. The epidemiology of leukoplakia is unclear due to the large variations in prevalence rates and the difficulties associated with conducting large epidemiological studies. Petti has estimated the pooled global prevalence of leukoplakia to be in the range of 1.49%–2.60% (5).

1.1.4 CANCEROUS TRANSFORMATION

In 2000, Hanahan and Weinberg proposed that carcinogenesis is characterised by six distinct cellular activities (6): sustained proliferation; evasion of growth suppression mechanisms; activation of invasion and metastasis; enabling of replicative immortality; induction of angiogenesis; and resistance to cell death. In 2011, the same authors updated this concept by adding the avoidance of immune destruction, tumour-promoting inflammation, genome instability and mutation, and deregulation of cellular energetics (7). Aberrant proliferation is

a fundamental activity of cancerous cells leading to cancer growth. In normal tissues, such as the healthy oral epithelium, tissue regeneration is tightly regulated, starting with the division of basal keratinocytes and proceeding with cell maturation and migration upwards in the epithelium (8). In a pre-malignant disorder such as leukoplakia, a disturbance of normal cell proliferation occurs, especially if dysplasia is present. Thus, dysregulation of cell proliferation can be considered as a hallmark of leukoplakia. This dysregulation is most probably a major cause of both leukoplakia recurrence after surgery and cancerous transformation.

1.1.5 RATE OF CANCEROUS TRANSFORMATION OF LEUKOPLAKIA

In a recently published meta-analysis covering 17,830 leukoplakias, the malignant transformation rate was 9.5% and the cumulative malignant transformation rate was 8.6%, with a yearly malignant transformation rate of 1.56% (9).

Several clinical and histopathological factors are used in the risk assessment of leukoplakia. Risk factors that have been correlated with malignancy include the clinical diagnosis of non-homogeneous leukoplakia; lesion size >200 mm^2; location on the tongue and/or the floor of the mouth; female gender; and the presence of epithelial dysplasia (10-13). Even when only one of these risk factors is present the transformation rate can be as high as 34% (14).

1.1.6 TREATMENTS FOR LEUKOPLAKIA

Several treatment protocols for leukoplakia have been suggested over the last few decades. However, the important question remains as to whether the clinical interventions are beneficial for patients with in terms of reducing their risk of cancer. In 2009, van der Waal presented a, now well-established, treatment algorithm for leukoplakia (13). In this algorithm, the diagnostic process starts with the exclusion and/or elimination of other possible causes for the white patch in the oral mucosa. If the lesion persists after interventional measures, a biopsy is recommended. The combination of histopathological examination and other risk factors is used to make decisions as to further surgical treatment and the surveillance interval (13).

The treatments modalities proposed for leukoplakia are excision of the lesion by conventional surgery or laser surgery, cryotherapy, photodynamic therapy or pharmacological treatment (15). The gold standard of treatment is surgical excision of the lesion, followed by surveillance with regular check-ups. If surgical excision is not feasible, several incision biopsies (mapping) with surveillance and surveillance alone are the second and third options of choice (11, 13, 16). Regardless of the above-mentioned approaches, cessation of risk-increasing activities, such as the use of tobacco and alcohol, is recommended.

1.1.7 RECURRENCE OF LEUKOPLAKIA AFTER SURGICAL TREATMENT

Despite complete surgical excision of the lesion, recurrence is a commonly encountered clinical problem, and recurrence rates in the range of 13%–42% have been reported (10, 11). The reason for lesion recurrence after surgery is unknown but is independent of the surgical excision method used.

1.1.8 CLINICAL DILEMMA

Results from several observational studies indicate that the risk of malignant transformation is not eliminated even when the leukoplakia is completely excised. Despite surgical removal, cancer transformation occurs in 3%–11% of all cases after excision of leukoplakia. Neither is the risk of malignant transformation reduced when a passive approach is adopted for the treatment of leukoplakia (10, 11, 15). The wait-and-watch approach is based on the concept that when the leukoplakia is under constant surveillance, cancerous transformation can be detected and treated at an early stage (16). Clinicians need to rely on risk factors associated with malignant transformation of leukoplakia when assessing the risk.

1.1.9 AETIOLOGY

Although the true aetiology of leukoplakia is not known, genetic mutations in the keratinocytes play an important role (17). In addition, various risk factors, such as the use of tobacco and alcohol and viral infection, have been implicated in the development of leukoplakia (18)

Previous studies have suggested that genetic alterations that occur in keratinocytes are a causative factor for leukoplakia (19). These genetic alterations increase the risk of malignant transformation and have been proposed to be a driver of the tumorigenesis of leukoplakia. In this context, genetic alterations that influence cell cycle regulation, genomic stability, apoptosis and Loss of Heterozygosity (LOH) are of importance (17).

Another aetiological factor in leukoplakia that has been discussed is infection with human papillomavirus (HPV). HPV infection of leukoplakia has been suggested as a co-factor for malignant transformation (20).

1.2 HUMAN PAPILLOMAVIRUSES

Human papilloma virus has been a part of human evolution and is one of the oldest viruses known to cause infection in man. HPV infection can manifest itself in several ways depending on the HPV type and the tissue that is the target of infection. The clinical manifestations range from benign warts, Heck's disease, papillomas and condylomas to potentially malignant lesions and squamous cell carcinomas (21, 22). Ever since Zur Hausen posited a causative association between HPV infection and cancer of the cervix uteri in the 1970s, correlations between HPV infection and other squamous cell carcinomas have been discussed (21, 23, 24). In 1983, Syrjänen and co-workers presented one of the first lines of evidence for a linkage between HPV infection and OSCC (25).

1.2.1 HPV GENOME

HPV is a small, non-enveloped, double-stranded DNA virus that encodes two groups of genes. The viral genome can be divided in nine regions, each of which contains an open reading frame (ORF)(26). The nine genomic regions are divided into early (E) and late (L) genes (Figure 3)(21). The early genes (E1, E2, E3, E4, E5, E6 and E7) are needed for transcriptional regulation and viral replication. The late genes (L1 and L2) encode the viral capsid protein (27) (Table 1).

Table 1. Function of the protein transcripted by the different coding sequences, the early (E) or late (L) genes.

Gene/ORF	Function of the protein
E1	Viral replication
E2	Viral transcription and DNA replication
E4	Important in the virion release
E5	Stimulate cell proliferation, downregulates MHC Class I molecules, activates EGFR
E6	Important oncoprotein, inhibits the p53 and interacts with several host-cell proteins.
E7	Interact with and inhibits pRb. Important oncoprotein and together with E6 induces malignant transformation.
E8	Not yet a proven function
L1	Major capsid protein
L2	Minor capsid protein, important to viral entry into nucleus
References	(21, 26)

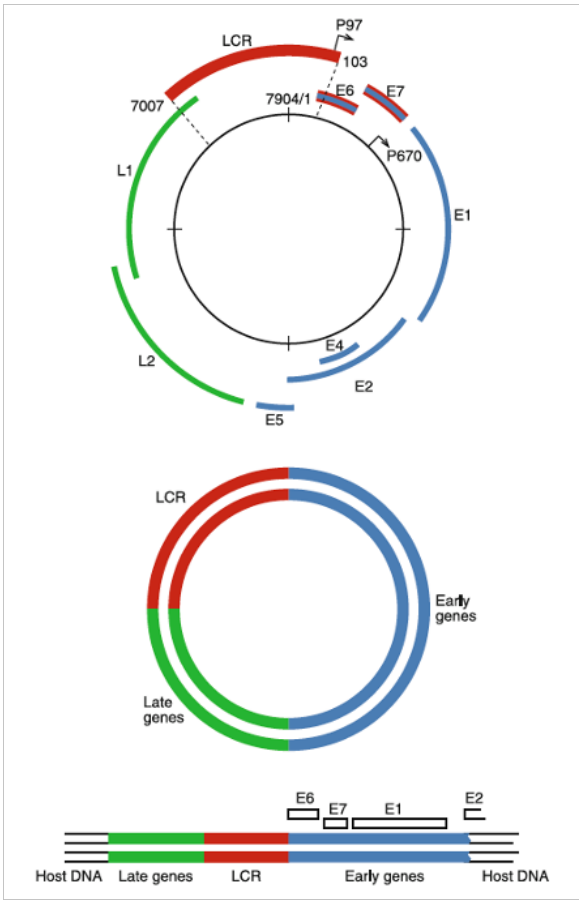

Figure 3. The HPV genome contains a circular double-stranded DNA inclosing the early (E) genes and late (L) genes together with a schematic picture of the integrated HPV DNA in the host genome. Reprinted from Rautava J and Syrjänen S. Biology of Human Papillomavirus Infections in Head and Neck Carcinogenesis. Head and Neck Pathol (2012) 6:S3–S15, DOI 10.1007/s12105-012-0367-2. With permission from Springer Nature (26).

1.2.2 CLASSIFICATION OF PAPILLOMAVIRUSES

Papillomavirus is a very diverse virus that infects both human and animals. It is divided into different taxonomic levels, building a phylogenetic tree (Figure 4). The taxonomic levels are as in descending order: Family, Genus, Species, Type, Subtype, and Variant (28).

The papillomavirus is classified based on the nucleotide sequence of the L1-gene, since it is the most conserved gene. The papillomavirus types belong to the same genus if they share less than 60% similarity. Different HPV types belong to the same species if they share 60%–70% similarity. Traditional papillomavirus types share between 71%-89% of the nucleotides within the L1 region. A subtype is defined as a difference in sequence homology of between 2% and 10% and a difference of <2% is defined as a variant (28).

A HPV type is classified as a novel subtype if it differs by more than 10% from the closest known papillomavirus (28). A novel HPV type is assigned a number when the whole genome has been cloned and a representative strain has been deposited in the International HPV Reference Center at the Karolinska Institute, Stockholm, Sweden.

Papillomaviruses are divided into 16 different genera and HPV belongs to 5 major genera: α-papillomaviruses, β-papillomaviruses, γ-papillomaviruses, μ-papillomaviruses, and ν-papillomaviruses (28). The most clinically interesting genus is the α-genus, since it high-risk HPV (HR-HPV) and low-risk HPV (LR-HPV) types belong to this group and infect mucosa (Figure 4). The virus is classified as a high-risk or low-risk HPV type depending on its ability to initiate malignant transformation of the infected tissue.

To date, 228 different HPV types have been listed and classified in the International HPV Reference Center.

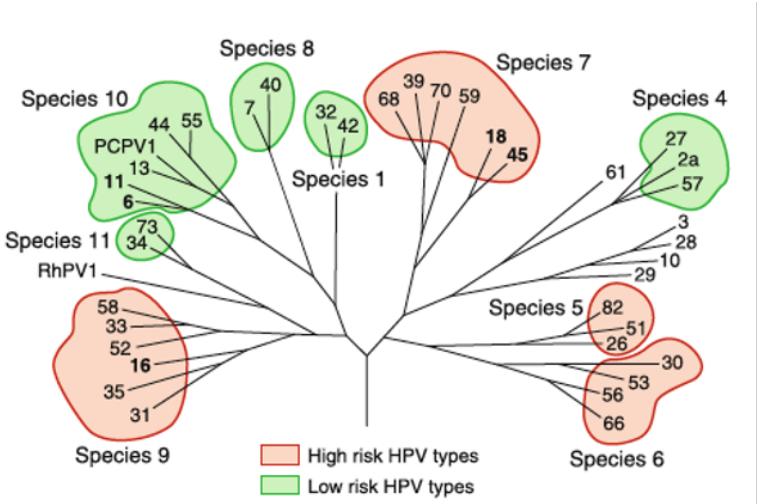

Figure 4. The phylogenetic tree of the α-genus divided in the high-risk and low-risk HPV types. Reprinted from Rautava J and Syrjänen S. Biology of Human Papillomavirus Infections in Head and Neck Carcinogenesis. Head and Neck Pathol (2012) 6: S3–S15, DOI 10.1007/s12105-012-0367-2. With permission from Springer Nature (26).

1.2.3 TRANSMISSION OF HPV

HPV infection is regarded as one of the most common sexually transmitted diseases, with a global prevalence of 11,7% (29). However, since mucosal HPV types have been found in the oral and genital mucosa of infants, virgins and children, there are also non-sexual transmission of HPV infection (21). Thus, Syrjänen and colleagues have presented strong evidence that HPV is also transmitted along a horizontal axis through the saliva and along a vertical axis from mother to child (21, 30).

1.2.4 HPV INFECTION

HPV is epitheliotropic, which means that HPV infects the epithelial cells, more precisely the basal layer in the stratified squamous epithelium. The virus needs to infect the proliferating basal keratinocytes in order to replicate. It reaches the basal cell often through a wound or a tear in the epithelium and enters the cell and then reaches the cell nucleus through mitosis mediated by L2 (late gene 2). When the viral DNA reaches the nucleus it is in the form of a plasmid.

During the initial stage of the infection, the viral DNA is replicated together with the cellular DNA in the keratinocytes. The HPV life-cycle is dependent upon the maturation of the host cell, such that the virus matures with the keratinocytes and migrates to the epithelial surface (26).

1.2.5 HPV-INDUCED CARCINOGENESIS

The mechanism underlying malignant transformation of the HPV-infected tissue is complex, with the key event in the malignant transformation being the integration of the HPV genome into the cell genome. The most-studied disease in which HPV causes cancer is carcinoma of the cervix uteri. Ninety percent of all cervical HPV infections are cleared, whereas 10% of HPV infections become persistent. A persistent HPV infection may cause intra-epithelial neoplasia which may develop into cervical carcinoma (31). Studies have revealed that HPV infections that persist for more than 12 months are associated with an increased risk of initiating a malignant transformation (32).

Integration of the HPV into the host genome may be followed by the expression of the viral oncoproteins E6 and E7 (33). E6 and E7 are important for both the induction and maintenance of the malignant process. E6 and E7 must persist in the epithelium to exert their oncogenic effects on the cell cycle, through the dysregulation of several important cellular regulatory proteins. The main effect of E6 and E7 is to interfere with the p53 protein and the retinoblastoma protein (pRb).

E6 binds to and inhibits p53, leading to inhibition of apoptosis. E7 binds to and inhibits the tumour suppressor protein pRb, blocking it from inactivating the transcription factor E2F, thereby causing the cell to enter cell division and to overexpress p16 (Figure 5) (33, 34) .

1.2.6 HUMAN PAPILLOMAVIRUS INFECTION OF THE HEAD AND NECK REGION

Ever since it was postulated in 1983 that HPV infection has a causative correlation to a subgroup of OSCC (25), the scientific community has produced numerous studies and meta-analyses on the subject, and the correlation has been debated intensively.

The prevalence rates of HPV in oral cavity, head and neck squamous cell carcinomas and PMOD have been reviewed in many studies and meta-analyses. The results reveal a large variations with regards to both anatomical sites and geographical regions (35, 36).

1.2.6.1 OROPHARYNGEAL SQUAMOUS CELL CARCINOMA AND HPV INFECTION

The role of HPV in head and neck squamous cell carcinoma (HNSCC) has been studied since the 1980s and the correlation between HPV and oropharyngeal squamous cell carcinoma (OPSCC) has been confirmed. HPV has been recognized as a risk factor for OPSCC by the WHO (2). Since the aetiology of OPSCC is considered to be multifactorial, infection with an HR-HPV alone is not sufficient to induce malignant transformation. The combination of an infection with an HR-HPV and high levels of tobacco and alcohol consumption leads to an overall increased risk for developing OPSCC (35, 37)

During the last two decades, there has been an alarming increase in the incidence of oropharyngeal carcinomas related to HPV infection (38-40).

1.2.6.2 ORAL SQUAMOUS CELL CARCINOMA AND HPV INFECTION

The correlation between HPV and OSCC has been described by several groups (22, 35). In a recent review and meta-analysis, the overall prevalence of HPV in OSCC was in the range of 13.4%–58.0%. HPV16 is the HPV type found most frequently in cases of OSCC, with prevalence rates of 50%–60% in the Asia-Pacific region, 40% in China, and 10.7% in Europe (35). In the same review, Syrjänen and Syrjänen reported a distinct geographical pattern, with the highest HPV prevalence in China and the lowest in North America (35).

1.2.6.3 LEUKOPLAKIA AND HPV INFECTION

The association between cervical or oropharyngeal cancer and HPV infection is well-known. Although a casual association between leukoplakia and HPV has been suggested in several reports, conclusive evidence has not been presented (20, 41, 42).

A recent extensive systematic meta-analysis of HPV prevalence in PMOD (36) revealed that the overall HPV prevalence in leukoplakia was 20.2%. The analysis was based on 28 studies and a total of 1,232 samples (36). When the analysis was restricted to HPV-positive leukoplakia and the HPV types were investigated, the most-prevalent HPV types that infected leukoplakia were

found to be HPV18 followed by HPV16 and HPV11 with rates of 64.5%, 40.8%, and 32.3% respectively. When they looked at the HPV prevalence in dysplastic versus non-dysplastic lesions, they found that the HPV prevalence in non-dysplastic lesions was 32.8% and in the dysplastic lesions it was 17.6%, resulting in a non-significant difference between the groups. Among the studies that contained a control group, the HPV prevalence in the control group was 10.3% and in the PMOD group it was 27.3% (36).

1.2.7 GLOBAL VARIATIONS IN HPV PREVALENCE

The systematic meta-analysis carried out by de la Cour and colleagues shows that the HPV prevalence rates differ across the continents: 16.5% in Asia, 25.0% in Europe, 26.6% in North America, and 46.8% in South America (36).

The detection rate for HPV in oral samples, including those from healthy controls and cases of leukoplakia, PMOD and OSCC, varies from 0% to 69% (42, 43). While there may be several reasons for this variability, the main ones appear to be the sampling methods and HPV detection methods used.

1.2.8 DETECTION OF HPV INFECTION

There are many different methods for detecting HPV infection. The choice of method depends on the type of sample and the tissue being analysed. HPV cannot be cultured in conventional cell cultures. However, it is possible to determine HPV infection by assessing the viral DNA, surrogate markers, HPV-specific antibodies or viral transcripts.

In clinical routine practice, the Papanicolaou test (PAP test) has traditionally been the main diagnostic method for detecting HPV in cytological smears from the cervix uteri. Light microscopy is still used in the clinical setting to detect HPV infection through observations of cellular abnormalities in the tissue, e.g., dyskeratosis, parakeratosis and koilocytosis. This technique is used in cytology and with biopsies taken from the uterine cervix. For samples collected from the oral cavity, this method is not reliable because in the histopathological picture of mechanical irritation vacuolated epithelial cells is often found and this might disguise the identification of true koilocytes (21, 44). HPV can be detected in various types of samples, such as in saliva, blood and biopsies (formalin-fixed and paraffin-imbedded, fresh or frozen tissue).

The detection of an HPV infection is possible through assaying for anti-HPV antibodies in the serum or saliva. The value of HPV detection through measuring anti-HPV antibodies is debated. The rate of detection of anti-HPV antibodies in the cervix uteri after a natural HPV infection is only 50%–70% of the cases (45, 46). Therefore, serological assays for the detection of HPV-specific antibodies have limitations in terms of analytical accuracy.

1.2.8.1 THE P16 PROTEIN AS A SURROGATE MARKER FOR A HPV INFECTION

The tumour-suppressor protein p16 is a well-established surrogate marker for HPV infection of the oropharyngeal mucosa and OPSCC. This protein is used in both clinical settings and scientific protocols to detect HPV in tissue´s and to predict outcomes for the cancer (47, 48).

In several studies, p16 has been proposed as a surrogate marker for HPV infection in the oral mucosa (49-51). However, there is no conclusive evidence of a correlation between HPV infection of the oral mucosa and the expression of p16 (52-54).

The p16 protein (also known as $p16^{INK4a}$ or cyclin-dependent kinase inhibitor 2A) is transcribed by the *CDKN2A* gene located on chromosome 9p21, together with the tumour-suppressor protein p14 (Figure 5). It works as an inhibitor of cell cycle progression by interfering with the pathway of the retinoblastoma protein (pRb) (55). The p16 protein inhibits the binding of the CDK4/6–cyclin D1 complex, resulting in inhibition of pRb phosphorylation. The phosphorylation of pRb leads to the release of the transcription factor E2F. E2F allows the cell to progress in the cell cycle and enter the S-phase. E2F also upregulates the transcription of p16 (55-57).

Infection with an HR-HPV and transcription of the viral oncoprotein E7 can disturb this pathway through E7 binding and inactivation of the pRb. This leads to the release of E2F, allowing cell cycle progression and for p16 expression to be upregulated (55).

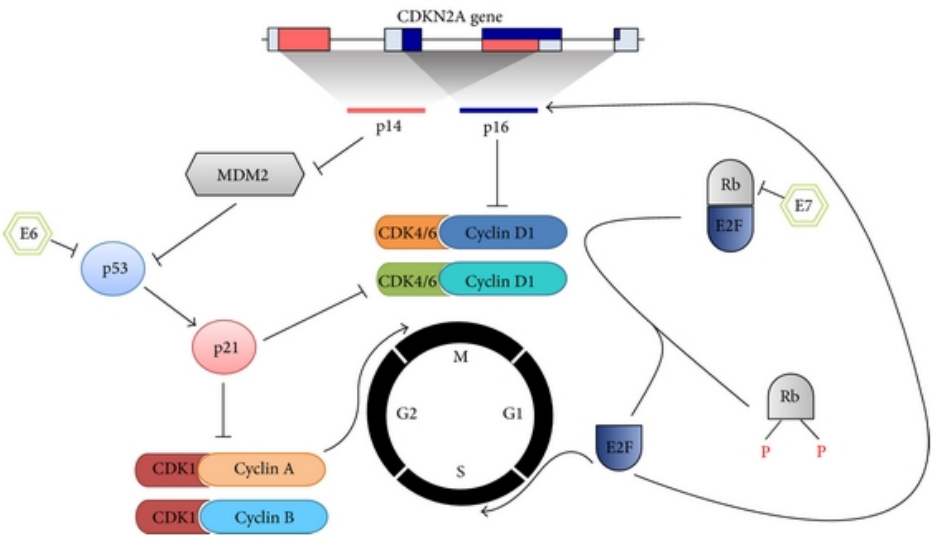

Figure 5. Cell cycle arrest by CDKN2A gene and the tumour suppressor protein p16 and p14. Reprinted with permission from Hindawi Publishing Corporation. A. Al-Kaabi et al. Disease Markers, vol. 2014, Article ID 260549, 8 pages, 2014. doi:10.1155/2014/260549

1.2.8.2 POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is used for the detection of both viral DNA and mRNA. PCR can be used with various types of samples, e.g., blood, saliva, oral swabs, cervical smears and biopsies (fresh, frozen or formalin-fixed and paraffin-embedded tissues).

PCR is a highly sensitive method. A limitation to the method is that when the PCR detects viral DNA it will also detect non-transcriptionally active viruses, i.e., a “bystander” phenomenon. To avoid this phenomenon, PCR can be used to detect the mRNA species for the viral oncoproteins E6 and E7 in the tissue. Positive PCR detection of the mRNA reveals infection with a transcriptionally active virus.

1.2.8.3 IN-SITU HYBRIDISATION

In-situ hybridisation (ISH) is another highly sensitive method for detecting HPV infection. ISH uses probes to identify and detect HPV sequences that are incorporated into the cellular DNA. ISH can detect viral oncogenic mRNA species for the 16 HR-HPV types and has the advantage that it detects

transcriptionally active viruses. ISH has the additional advantage that it visualises the HPV infection in the tissue and distinguishes it from the tissue morphology. However, when viral DNA levels are low, ISH may have difficulties in detecting the HPV infection (58).

1.3 RISK ASSESSMENT FOR LEUKOPLAKIA

There are ongoing efforts to identify molecular markers or diagnostic tests that will define a leukoplakia that will undergo malignant transformation. These efforts are complicated by the fact that clinical and histopathological data alone cannot predict whether leukoplakia will remain stable, regress or develop into an OSCC.

The hunt for a reliable biomarker or satisfactory diagnostic test has taken many paths. Loss of heterozygosity (LOH) is one of the most investigated areas. (17). Other areas investigated for their value in risk assessment for leukoplakia are cell cycle deviations, DNA ploidy status, different epithelial growth factors, cell surface glycoproteins and prostaglandin synthesis (17).

Biomarkers for detecting recurrence of leukoplakia after surgical excision and its relation to malignant transformation of leukoplakia are considered in this thesis, with the focus on p53, p63, podoplanin (PDPN) and Ki-67.

1.3.1 TUMOUR SUPPRESSOR PROTEIN P53

The transcription factor and tumour suppressor protein p53 is encoded by the *TP53* gene. The p53 protein: 1) regulates the transition from the G1 phase to the S phase in the cell cycle; 2) prevents mutations in the genome by detecting DNA aberrations; and 3) is involved in cell cycle control by promoting cell cycle arrest and apoptosis in response to cellular stress. It is also a key player in the preservation of genomic stability, including DNA repair (59). Thus, p53 is labelled “the guardian of the genome”, and some form of inactivation of p53 occurs in the majority of the human cancers. Suppression of p53 by the occurrence of mutations in the *TP53* gene is commonly seen in OSCC, and somatic mutation of *TP53* is detected in 60%–80% of all the head and neck cancers (60).

Under normal conditions, wild-type p53 is detectable at very low levels in the oral epithelium, owing to its negative feedback loop (61). A mutated form of p53 has an extended half-life and can, therefore, be detected at higher levels in pathologically altered epithelia (62). Therefore, overexpression of p53 might indicate a mutation in the *TP53* gene.

The p53 protein has been extensively studied as a biomarker for the detection of both dysplasia and malignant transformation of leukoplakia (63-65). Mello and co- workers demonstrate in recent published review article, where they

investigated prognostic biomarkers in PMOD, that two out of four investigated studies show a significant correlation between high expression of p53 and malignant transformation of leukoplakia (66). Even though supra-basal expression of p53 correlates with transformation of leukoplakia to OSCC, assays of the expression of p53 alone do not have sufficient sensitivity and specificity to make it a useful candidate molecular marker for malignant transformation.

1.3.2 TUMOUR SUPPRESSOR PROTEIN P63

In 1997 the tumour suppressor protein p63 was initially described as keratinocyte transcription factor (KET). The tumour suppressor protein p63 is related to and closely connected with p53 (67). The p63 protein controls the development of the epidermis during embryogenesis (68), and it has been demonstrated to be an oncogenic driver in squamous cell carcinomas (69, 70).

The *p63* gene is located on chromosome 3q27–29 and is responsible for the transcription of two main isoform groups of the p63 protein, ΔNp63 and TAp63. These isoform groups each contain three different isoforms, are responsible for different functions in the cell cycle, and are highly cell-dependent (71). ΔNp63 is the most-common isoform group and is mainly expressed in the stratifying epithelium, where it preserves its capacity to promote proliferation (70).

The ΔNp63 group lacks the canonical N-terminal transactivation domain and acts by inhibiting both p53 and TAp63, leading to cell proliferation. The TAp63 isoform group contains the same canonical N-terminal transactivation domain and exerts functions similar to p53, such as cell cycle arrest and apoptosis (71, 72).

In contrast to TP53 the *TP63* gene is rarely mutated in human cancer , the overexpression of p63 is not due to a mutation in the gene; it is more likely that intracellular events leading to different cascades enhance its expression and transcriptional activity (70, 73).

In both oral dysplastic lesions and OSCC, p63 expression has been shown to be upregulated (65, 74, 75).

1.3.3 PODOPLANIN

PDPN is expressed in keratinocytes and is upregulated in basal keratinocytes under hyper-proliferative conditions, such as wound healing or when exposed to inflammatory stimuli (76).

PDPN is a mucin-type transmembrane glycoprotein that is generally expressed on the cell surfaces of different tissues and cells, such as lymphatic vessels, alveoli, heart tissue, macrophages, T-helper cells, and fibroblasts (77, 78). PDPN plays important roles in the immune response, platelet aggregation, and the development of multiple organs, such as the lungs, heart and lymphatic system (79). When PDPN is activated, it interacts with other proteins inside the same cell or in adjacent cells. The activation of PDPN leads to alteration of the signalling pathways that regulate cell proliferation, migration and contractility remodelling of the extracellular matrix (78).

In leukoplakia, PDPN has been proposed as a biomarker for increased risk of cancer. Recently, Aiswaryia and co-workers have shown that PDPN expression gradually increases with the grade of dysplasia and early OSCC (80). Correlations of PDPN with malignant transformation of leukoplakia and OSCC have previously been shown by different groups (81, 82). Even though a positive correlation between PDPN and malignant transformation of leukoplakia has been described (81), the sensitivity and specificity of a diagnostic test assessing PDPN is too low to screen out leukoplakia with risk for malignant transformation. Neither has PDPN as a biomarker been assessed systematically and prospectively in studies (17).

1.3.4 KI-67

Ki-67, which is a marker of cell proliferation, is used in the diagnostic setting for the histopathological evaluation of patients with premalignant or malignant disease.

The Ki-67 protein has been extensively studied in cases of OSCC and leukoplakia. Ki-67 is present in all active phases in the cell cycle (G1, S, G2 and mitosis) but absent from G0 (83). Expression of Ki-67 has been correlated to the grade of dysplasia and malignant transformation (84).

The pattern and intensity of Ki-67 expression have been correlated with both dysplastic oral lesions and malignant transformation of leukoplakia (64, 85).

While many different approaches have been taken and the methods used have been advanced and sophisticated, we are still stuck with the same question regarding leukoplakia. Is it possible to predict the outcome of the leukoplakia? So far, no diagnostic test or method with sufficient sensitivity or specificity has become available.

One way to proceed is to create an algorithm based on a combination of different molecular markers, genetic factors, and the clinical and histopathological information. Applying that algorithm, it will be possible to predict the outcome of every case of leukoplakia. This is the challenge that lies ahead.

2 AIM

The overall aim of this thesis was to investigate the influence of HPV infection in oral leukoplakia, which clinical-, histopathological and treatment factors that affect the recurrence rate after surgical removal and to investigate molecular markers connected to cell proliferation and cancer transformation.

The thesis is based on the following five scientific questions tackled in the respective studies

SCIENTIFIC QUESTIONS

Study I
Is there a correlation between overexpression of the tumour suppressor protein p16 and HR-HPV infection in leukoplakia and oral squamous cell carcinoma?

Study II
i) Has the HR-HPV prevalence in leukoplakia changed over time?
ii) Does the HR-HPV prevalence in leukoplakia differ between three geographical regions: Sweden, Romania and Brazil?

Study III
Which clinical and anamnestic factors correlate with the recurrence of leukoplakia after surgical removal?

Study IV
Can expression of the cell proliferation biomarkers p53, p63, podoplanin and Ki-67 predict the recurrence of leukoplakia after surgical excision?

3 PATIENTS AND METHODS

3.1 PATIENTS

The ORA-LEU-CAN study forms the basis for this thesis. We have requisitioned clinical information, anamnestic data and biopsy materials from the patients who are participating in this prospective study.

3.1.1 THE ORA-LEU-CAN STUDY

The ORA-LEU-CAN study is an ongoing prospective, longitudinal, multi-centre study conducted at five centres in Sweden:

Gothenburg

- Clinic of Oral Medicine
- Clinic of Oral and Maxillofacial Surgery
- Clinic of Oral Medicine East

Trollhättan

- Clinic of Orofacial Medicine - Norra Älvsborgs Länssjukhus

Uppsala

- Clinic of Oral and Maxillofacial Surgery - Uppsala University Hospital

Patients are referred to the participating centres from general dental practitioners, general medical practitioners and ear-nose-throat specialists.

In the ORA-LEU-CAN study, patients with leukoplakia are included and treated according to the standard of care. The inclusion criterion is a clinically verified diagnosis of leukoplakia. The participating centres define leukoplakia in accordance with the WHO criterion (1). A patient is followed over a 5-year period and the follow-up interval is every third month in the first 2 years and every sixth month in the subsequent 3 years.

In the study, anamnestic data, including gender, age, medical history, tobacco habits, alcohol habits, and medication, are collected. Moreover, information on the clinical and histopathological diagnoses, size of lesion, location, and

existence of single or multiple lesions, together with clinical photographs is registered. In the histopathological reports, epithelial dysplasia is scored according to the WHO classification scale (2).

3.1.2 STUDY I

Clinical data and tissue specimens from 95 patients in the ORA-LEU-CAN study, obtained between 2011 and 2017, were retrieved from the study database. Re-reviews of the clinical diagnoses by two specialists in oral medicine resulted in the exclusion of 14 patients, due to a revised diagnosis (N=10) or uncertainties related to the diagnosis (N=4). Thus, 81 patients were included in the final analysis.

Fifteen patients with OSCC and five patients who exhibited cervical carcinoma with confirmed expression of the tumour suppressor protein p16 were included, and their samples were retrieved from the Diagnostic Biobank at the Department of Clinical Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden.

After re-evaluation of the expression levels of p16, two patients with OSCC were considered to be p16-negative and were excluded. The five patients with cervical carcinoma were all p16-positive. This latter group of patients served as a positive control in the PCR analysis.

The characteristics of the patients with leukoplakia and OSCC are listed in paper I.

3.1.3 STUDY II

To determine whether HR-HPV prevalence changes over time and varies with geographical region, this study utilised six different cohorts: three contemporary cohorts and three historical cohorts. The patient cohorts were collated from Sweden, Romania and Brazil, and from two different time periods.

The Swedish contemporary cohort was retrieved from the ORA-LEU-CAN study for the period 2011–2017. For the Swedish historical cohort, patients with leukoplakia were retrieved through a registry search of the database at the

Department of Oral Medicine and Pathology, Institute of Odontology, University of Gothenburg, for the time period 1992–2002.

In Romania and Brazil, the contemporary (covering the period 2011–2017) and the historical (1992–2002) cohorts were both obtained after registry searches of the databases at the Departments of Oral Medicine and Pathology at the Sao Paulo and Bucharest universities.

The inclusion criterion was a clinical diagnosis of leukoplakia, verified according to the medical records and referral documents. The exclusion criteria were a history of head and neck cancer, tissue specimens of less than 5 mm in diameter, and incomplete medical records. In total, 432 patients from the three countries were included in the analysis. The contemporary cohorts contained 100 patients from Sweden, 35 patients from Romania, and 100 patients from Brazil. In the historical cohorts, there were 89 patients from Sweden, 19 from Romania, and 89 from Brazil. The patients' characteristics are listed in Table 2.

Table 2. Demographic and clinico-pathological characteristics of the patients with leukoplakia from the different cohorts (86).

Country	**Sweden N (%)**		**Brazil N (%)**		**Romania N (%)**	
Cohort	**Historical N = 89**	**Contemporary N = 100**	**Historical N = 89**	**Contemporary N = 100**	**Historical N = 19**	**Contemporary N = 35**
Gender						
Male	40 (45)	54 (54)	50 (56)	52 (52)	7 (37)	10 (29)
Female	49 (55)	46 (46)	39 (44)	48 (48)	12 (63)	25 (71)
Age (years)						
Mean (median)	59 (58)	60 (61)	58 (56)	57 (46)	55 (53)	60 (61)
Range	20–91	27–82	32-90	35-89	30–79	42–86
Site of lesion						
Floor of the mouth	17 (19)	7 (7)	10 (11)	15 (15)	4 (21)	1 (3)
Buccal mucosa	26 (29)	22 (22)	18 (20)	16 (16)	2 (11)	7 (20)
Tongue	14 (16)	34 (34)	35 (40)	38 (38)	7 (37)	6 (17)
Gingiva	25 (28)	31 (31)	20 (23)	24 (24)	4 (21)	17 (49)
Hard palate	6 (7)	3 (3)	3 (3)	2 (2)	1 (5)	4 (11)
Soft palate	1 (1)	3 (3)	3 (3)	5 (5)	1 (5)	0 (0)
Dysplasia						
Yes	19 (21)	25 (25)	62 (70)	71 (71)	6 (32)	13 (37)
No	70 (79)	75 (75)	27 (30)	29 (29)	13 (68)	22 (63)

When the study cohorts were established, a second registry search was made in each centre, to determine whether the leukoplakia seen in the included patients had transformed into OSCC.

3.1.4 STUDY III

Patients included in the ORA-LEU-CAN study in the period between 2011 and 2018 formed the study cohort in Study III. A minimum of 6 months of follow-up was used as an inclusion criterion.

In total, 226 patients were recruited. After review of their clinical data, 46 patients were excluded due to revision of the clinical diagnosis and 20 patients were excluded due to a concomitant other oral disorder(s). In eleven patients, the histopathological examination of the biopsy revealed an OSCC already at the time of inclusion. Thirteen patients had incomplete records and two patients developed OSCC at another location in the oral cavity. Ultimately, 180 patients proceeded to the analysis.

A recurrence of leukoplakia was defined as the reappearance of leukoplakia at the same site as that of the primary leukoplakia. A clinically healthy mucosa had to be recorded with a clinical photograph between the time of surgery and recurrence (Figure 6). The patients' characteristics are listed in paper III.

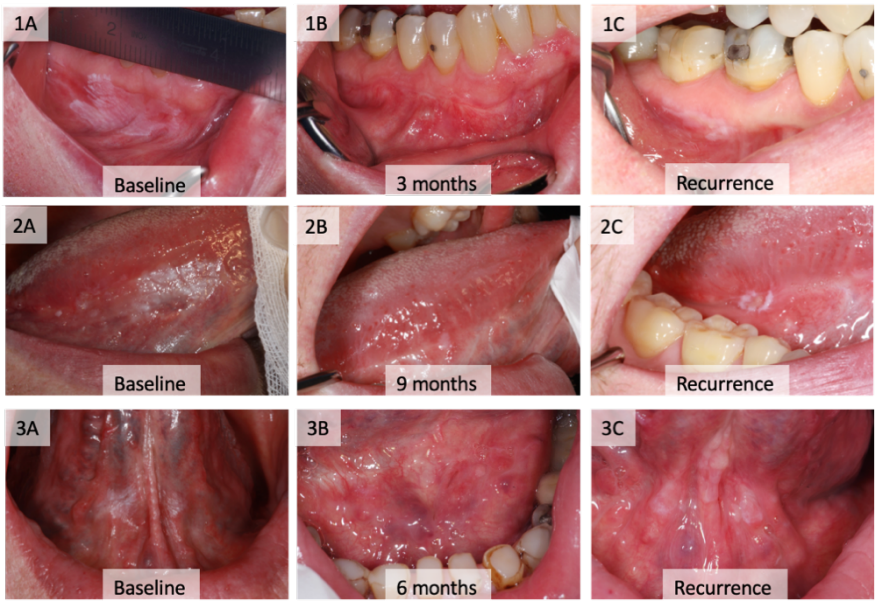

Figure 6. Clinical appearance of three patients with leukoplakia (patient 1,2 and 3). A clinically healthy mucosa had to be recorded with a clinical photograph between the time of surgery and recurrence. Printed with permission from PLoS ONE. Sundberg J, Korytowska M, Holmberg E, Bratel J, Wallström M, Kjellström E, et al. (2019) Recurrence rates after surgical removal of oral leukoplakia—A prospective longitudinal multicentre study. PLoS ONE 14(12): e0225682. https://doi.org/10.1371/journal.pone.0225682

3.1.5 STUDY IV

In Study IV, 80 patients who were included in the ORA-LEU-CAN study during the period 2011–2018 were retrieved from the study database. The inclusion criteria were a clinically verified leukoplakia that was surgically removed with margin and at least 6 months of follow-up.

Clinical data and photographs and the results of histopathological examinations were extracted from the database. Epithelial dysplasia was histopathologically scored according to the WHO classification scale (2). For the presence of dysplasia, a binary scale was used, i.e., no dysplasia or dysplasia.

The clinical diagnoses were reviewed by two specialists in oral medicine. For seven patients, tissue specimens were not available. Thus, 73 patients proceeded to the analysis. The patients' characteristics are listed in paper IV.

3.2 ETHICAL CONSIDERATIONS

The studies were approved by the following Ethical Review Boards:

Regional Ethics Review Board in Gothenburg: *Premalignant and malignant disease states in oral, clinical, immunological, genetic and viral aspects of diagnosis and treatment* - EPN Gbg Dnr. 673-10 / T872-18, DNR T644-10 / 618-05.

Ethical Board at the School of Dentistry, University of Sao Paulo, Brazil (protocol no. 2.201.757).

Ethical Review Board at the Institutul National de Cercetare-Dezvoltare in Domeniul Patologiei si Stiintelor Biomedicale “Victor Babes”, Bucharest, Romania (protocol no. 54:06.12.2017).

These studies were conducted in accordance with the Declaration of Helsinki.

3.3 LABORATORY TECHNIQUES

The main laboratory techniques utilized in the work of this thesis were immunohistochemistry (IHC) and PCR analysis. IHC and PCR were performed In Study I, PCR was performed in Study II, and IHC was used in Study IV.

3.3.1 IMMUNOHISTOCHEMISTRY

3.3.1.1 STUDY I – TUMOUR SUPPRESSOR PROTEIN P16

From formalin-fixed and paraffin-embedded (FFPE) tissue samples, 4-µm-thick sections were prepared. The tissue sections were stained with a murine monoclonal antibody against p16 (clone E6H4, CINtec Histology Kit; Roche Diagnostics GmbH, Mannheim, Germany). Tissue from a tonsillar cancer was used as a positive control and the omission of the primary antibody was used as a negative control.

3.3.1.2 STUDY IV – BIOMARKERS P53, P63, KI-67 AND PODOPLANIN

FFPE tissue samples from cases of leukoplakia were sliced into 4-µm-thick sections, which were placed on positively charged glasses.

For the detection of p53, p63, Ki-67 and PDPN in IHC, the following antibodies were used:

- For P53 – Monoclonal mouse anti-human p53 primary antibody (M700101-2, clone DO-7; DAKO, Glostrup, Denmark)
- For P63 – Polyclonal rabbit anti-human TP63 antibody (anti-TP63, HPA006288; Atlas Antibodies AB, Bromma, Sweden)
- For Ki-67 – Monoclonal mouse anti-human Ki-67 (clone MIB-1; DAKO)
- For PDPN – Monoclonal mouse anti-human podoplanin antibody (M361901-2, clone D2-40; DAKO)

Sections from tonsils and OSCC served as positive controls, while omission of the primary antibodies served as negative controls.

3.3.2 CELL QUANTIFICATION

3.3.2.1 STUDY I

In Study I, the expression of p16 was recorded and assessed using a semi-quantitative scale based on the percentage of p16 expression in the oral epithelium. A semi-quantitative scoring scheme was used that had a range from one to five, as described in detail in Paper I. A sample was regarded as positive when it had a score ≥3 (Figure 7).

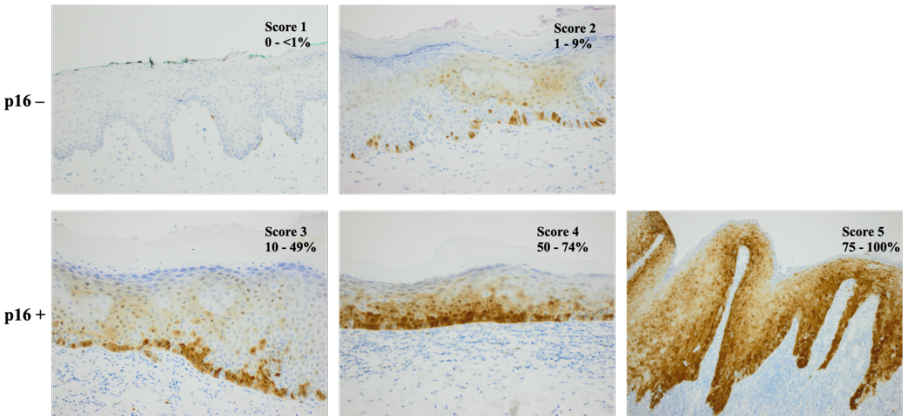

Figure 7. "Semi-quantitative assessment of p16 expression based on the percentage of p16 expression detected in the epithelial layer, using the following scoring scheme: 1, 0–1%; 2, 1–9%; 3, 10–49%; 4, 50–74%; and 5, 75–100%. Samples with scores in the range of 3–5 were regarded as p16-positive"(87).

3.3.2.2 STUDY IV

All the slides with tissue sections were scanned using the NanoZoomer XR digital scanner (Hamamatsu Inc., Hamamatsu-city, Japan) at 40× magnification. The patterns of expression of p53, p63 and Ki-67 in leukoplakia specimens were recorded and evaluated using the QuPath open source software for digital pathology image analysis (88). The expression of PDPN was recorded and evaluated manually under a light microscope.

Each section of the slide was divided into five regions of the epithelium: three in the centre (randomly chosen) and one in each resection margin (Figure 8A). The margin was analysed only when it was identified with confidence. When it was difficult to define the margin, only the centre parts were analysed. A margin was assessed as confident when the sample had a clear-cut margin without any artefacts, such as folding, tissue tears or non-specific staining. When there was an artefact within the tissue sample the closest part of the artefact was analysed. To avoid the risk for misleading results, the stratum corneum was excluded in the analysis.

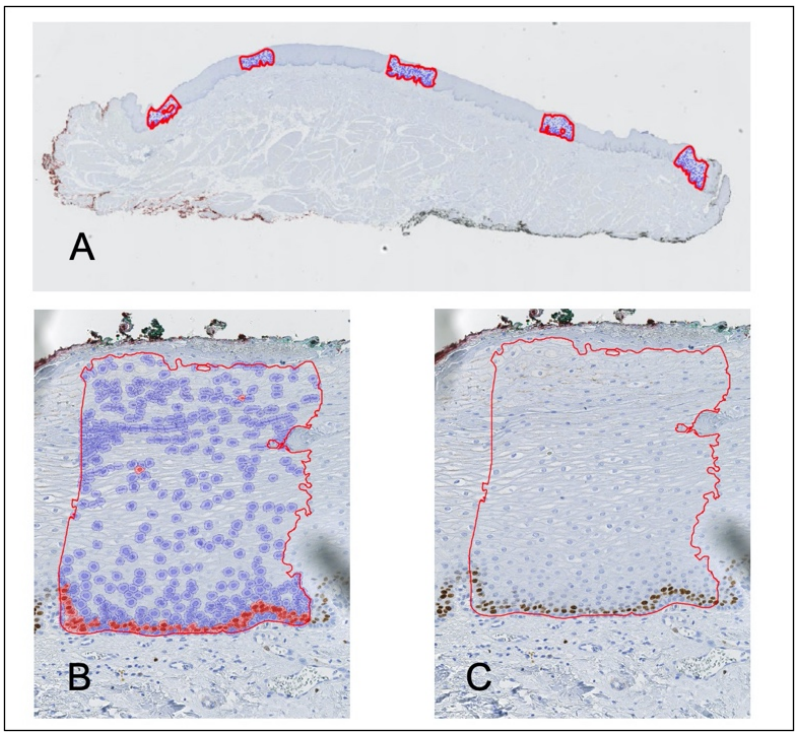

Figure 8. A; A section divided into five parts of the epithelium, three in the center and one in each resection margin. Area selected was 50,000 to 100,000 µm² with minimum 200 cells included for each region. B + C; illustrate the validation process of the Qupath software. B shows the detected cells, both positive and negative cells and C illustrate the same area without detected cells.

Before proceeding with digital scoring, a validation of the detection capacity of the program (QuPath software) was carried out (88) (Figure 8B+C). For this, 10 tissue samples were randomly selected. Manual detection of positive and

negative cells was performed, followed by automated analysis of the same area. Both the automated and manual scoring results were documented and compared, and this was followed by a comparison and an evaluation of the results. As the setting was optimum, it was maintained for all the samples. For PDPN, brown granular membranous staining of the epithelial cell layer was considered to indicate positivity. The percentage of PDPN-positive cells was calculated. All cell counting was performed in a blinded fashion and the clinical information was revealed after the analysis was completed.

3.3.3 POLYMERASE CHAIN REACTION

For Studies I and II, PCR analyses were performed to detect HPV-DNA in the samples. To determine the presence of HPV-DNA in the tissue, the DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were analysed in a spectrophotometer (DropSense96; Unchained Labs, Pleasanton, CA, USA).

A TaqMan Real-Time PCR assay targeting 12 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) and 2 low-risk (6 and 11) types of HPV was used to identify the HPV DNA in the samples. The primers and probes are presented in Study I and Study II, and the PCR method used has been published previously (89).

In parallel with the HPV analysis, a Real-Time PCR analysis of the human *β-globin* gene was run as a quality control for each sample. The set cycle threshold (C_t)-value for the *β-globin* gene was a maximum of 40 cycles. A tissue sample with a C_t-value >40 was considered as insufficient, and that patient was excluded from the study.

As a positive control, a plasmid (pUC57) that contained target segments of all the investigated HPV types was used to verify the performance of the PCR for each HPV type. As an extra positive control, HPV-positive samples of cervical cancer and OSCC were added in the analyses.

3.4 STATISTICAL ANALYSES

3.4.1 STUDY I

Analyses of differences between groups were performed using Fisher's exact test. A p-value < 0.05 was considered statistically significant.

3.4.2 STUDY III AND STUDY IV

Recurrence of leukoplakia versus no recurrence of leukoplakia was the primary outcome in our analysis. The follow-up time was defined as the time from the first excision surgery to the time of recurrence or to the last visit within the study protocol. In case of death before a recurrence, the death was censored (this was the case for one patient). A recurrence was defined as an event in the Kaplan-Meier analysis and in the Cox regression analysis.

In Study III, the cumulative, disease-free survival rates were calculated using Kaplan-Meier survival curves, and the outcomes for patients in the different group were compared using a two-sided log-rank (Mantel-Cox) test.

In Study IV, Kaplan-Meier estimates were used to construct diagrams showing the cumulative incidence of leukoplakia stratified by the expression levels of the biomarkers, with a cut-off above or below the median value of the expression.

In Study III and Study IV, univariable and multivariable Cox regression analyses were utilized to identify risk factors for predicting the recurrence of leukoplakia. The analyses were based on the expression of biomarkers as continuous variables and dichotomised clinical factor. The effects of the analysed factors were described as a Hazard Ratio (HR) with 95% confidence interval (CI). Assumptions of proportional hazards were tested using Schoenfeld's residuals. All the variables in the Cox regression analyses fulfilled the assumption for both Study III and Study IV. A p-value <0.05 was considered statistically significant.

Statistical analyses were carried out using the SPSS Statistics for Macintosh ver. 25.0 software package (IBM Corp., Armonk, NY, USA), Stata Statistical Software 2019: release 16; StataCorp LLC, College Station, TX, USA), and Prism 8 software (GraphPad Software Inc., San Diego, CA, USA).

4 RESULTS

4.1 STUDY I

A quality check of the amount of DNA extracted from the tissue samples was performed using the human *β-globin* gene as a control. Overall, 7/81 patients were excluded due to an insufficient amount of DNA, which meant that 74 patients in the leukoplakia group proceeded to the analyses. The p16-positive OSCC and the p16-positive cervical carcinoma displayed sufficient amount of DNA for PCR analysis.

Does overexpression of the tumour suppressor protein p16 correlates with high-risk HPV infection in leukoplakia?

The overexpression of p16 was observed in 13/74 (18%) of the patients with leukoplakia. No patient in the leukoplakia group was positive for any of the investigated HPV types (Table 3).

There were no significant correlations between the overexpression of p16 and the clinical diagnosis, presence of dysplasia, gender of the patient, smoking or use of snuff ($p = 0.74$, $p = 0.25$, $p = 0.76$, $p = 0.52$, $p = 0.28$, respectively) (Table 3).

Expression of p16 and cancer transformation

None of the p16-positive leukoplakias transformed into OSCC, while four patients (7%) with p16-negative samples transformed to OSCC (Table 3).

High-risk HPV infection and overexpression of p16 in OSCC

Five (of the total of 13) patients with p16-positive OSCC (38%) were positive for the investigated HR-HPV types.

High-risk HPV infection in patients with cervical carcinoma

In this control group, four out of five patients (80%) with p16-positive cervical carcinoma were positive for the investigated HR-HPV types.

Table 3. Clinical characteristics and the p16 expression in leukoplakia and OSCC (87).

Patients	Leukoplakia, N (%)		OSCC, N (%)
	p16-Positive (N=13)	p16-Negative (N=61)	p16-Positive (N=13)
Gender			
Male	7 (54%)	29 (48%)	9 (69%)
Female	6 (46%)	32 (52%)	4 (31%)
Cancer transformation	0 (0%)	4 (7%)	-
HPV PCR result			
Positive	0 (0%)	0 (0%)	5 (38%)
Negative	13 (100%)	61 (100%)	8 (62%)
Clinical diagnosis			
Homogenous	10 (77%)	42 (69%)	-
Non-homogenous	3 (23%)	19 (31%)	-
Dysplasia			
Yes	4 (31%)	10 (16%)	-
No	9 (69%)	51 (84%)	-
Site of lesion			
Tongue	2 (15%)	19 (31%)	2 (15%)
Buccal mucosa	3 (23%)	12 (20%)	0 (0%)
Gingiva	8 (62%)	19 (31%)	8 (62%)
Floor of the mouth	0 (0%)	5 (8%)	1 (8%)
Palate	0 (0%)	3 (5%)	2 (15%)
Lip	0 (0%)	3 (5%)	0 (0%)

4.2 STUDY II

Among the 432 patients, 14 patients, all from the Swedish cohorts, had a C_t-value >40 and were accordingly excluded. After this exclusion, 418 patients remained: 175 from Sweden, 189 from Brazil, and 54 from Romania (Table 4). Five out of 418 patients were positive for the investigated HR-HPV types (HPV 11,16,16,31,33, Table 4).

In total, 17 patients with leukoplakia developed OSCC at the same site as the leukoplakia (5 Swedish, 5 Brazilian, and 7 Romanian patients) were found (Table 5).

Table 4. Results of the Real-Time PCR analysis for detection of HR-HPV in leukoplakia in the periods 1992-2002 and 2011-2017 (86).

Cohort	Leukoplakia	Sweden	Brazil	Romania	Total
Historical 1992-2002	No. of patients	82	89	19	190
	HPV-positive (sub-types)	0	1 (11)	0	1
	HPV-negative	82	88	19	189
Contemporary 2011-2017	No. of patients	93	100	35	228
	HPV-positive (sub-types)	0	4 (16,16,31,33)	0	4
	HPV-negative	93	96	35	224

Has the high-risk HPV prevalence in leukoplakia changed over time?

In the three contemporary cohorts (2011 – 2017) four out of 228 patients were positive for the investigated HPV types (Table 4). One patient was positive for the investigated HR-HPV types among the 190 patients in the three historical cohorts (Table 4). No increase in prevalence of HR-HPV infection was found.

Does high-risk HPV prevalence in leukoplakia differ between Sweden, Romania and Brazil?

Of the 189 patients in the Brazilian cohort, 5 (3%) were positive for the investigated HR-HPV types 11, 16, 16, 31 and 33 (Table 4). In the Swedish and Romanian cohorts, none of the investigated HR-HPV types were detected (Table 4).

High-risk HPV infection and malignant transformation of leukoplakia

Overall, 17/418 (4%) patients with leukoplakia displayed cancerous transformation at the lesion site. In the Swedish historical and contemporary cohorts, five patients developed malignant transformation of their leukoplakia (Table 5). The corresponding numbers in the Brazilian and Romanian cohorts were five and seven patients, respectively. The HR-HPV types were not detected in any of the 17 primary leukoplakia that developed into OSCC.

Table 5. Characteristics and HPV status of the patients with leukoplakia that transformed into OSCC (86).

Patient No.	Age at leukoplakia diagnosis (years)	Gender	Site of leukoplakia	Presence of dysplasia	HPV status	Time (months) to cancer transformation	Site of cancer	HPV status	Country	Cohort
1	27	Female	Tongue	Yes	Neg	33	Tongue	Neg	Sweden	Contemporary
2	61	Female	Tongue	Yes	Neg	11	Tongue	Neg	Sweden	Contemporary
3	65	Female	Tongue	No	Neg	54	Tongue	Neg	Sweden	Contemporary
4	52	Male	Gingiva	No	Neg	47	Gingiva	Neg	Sweden	Contemporary
5	54	Female	Tongue	No	Neg	26	Tongue	Neg	Sweden	Contemporary
6	72	Female	Buccal mucosa	Yes	Neg	24	Buccal mucosa	Neg	Romania	Contemporary
7	56	Female	Tongue	Yes	Neg	30	Tongue	Neg	Romania	Contemporary
8	30	Female	Tongue	No	Neg	15	Tongue	Neg	Romania	Historical
9	63	Female	Gingiva	No	Neg	60	Gingiva	Neg	Romania	Contemporary
10	76	Female	Tongue	Yes	Neg	48	Tongue	Neg	Romania	Historical
11	66	Female	Gingiva	Yes	Neg	48	Gingiva	Neg	Romania	Contemporary
12	63	Female	Hard palate	Yes	Neg	36	Hard palate	Neg	Romania	Contemporary
13	80	Female	Tongue	Yes	Neg	36	Tongue	Neg	Brazil	Historical
14	81	Female	Gingiva	Yes	Neg	22	Gingiva	Neg	Brazil	Historical
15	72	Female	Buccal mucosa	Yes	Neg	24	Buccal mucosa	Neg	Brazil	Contemporary
16	69	Female	Buccal mucosa	Yes	Neg	30	Buccal mucosa	Neg	Brazil	Contemporary
17	70	Female	Floor of the mouth	Yes	Neg	15	Floor of the mouth	Neg	Brazil	Contemporary

4.3 STUDY III

Of the 180 patients with leukoplakia who were included during the study period, 103 underwent complete surgical excision and 43 (42%) had recurrence of leukoplakia at the primary lesion site. The cumulative incidences of recurrence after 4 and 5 years were 45% (95% CI 35%–56%) and 49% (95% CI 28%–60%), respectively. The median follow-up time to recurrence was 1.3 years (min–max: 0.2–5.7 years), and to the end of follow-up for those censored it was 4.0 years (min–max: 0.9–6.0 years).

Which clinical and anamnestic factors correlate with recurrence of leukoplakia after surgical excision?

The factors that showed significant correlations to recurrence after surgical excision were clinical diagnosis of non-homogenous leukoplakia, and use of Swedish snuff.

Non-homogeneous leukoplakia recurred more frequently than homogenous leukoplakia ($p = 0.021$) and leukoplakia in patients who used snuff recurred more frequently than leukoplakia in patients that did not use snuff ($p = 0.003$) (Table 6). A clinical diagnosis of non-homogenous leukoplakia doubled the risk for recurrence (HR:2.00, 95% CI 1.10–3.65; $p = 0.024$), as compared with a homogenous leukoplakia (Table 7). Use of snuff was associated with a 3-fold increased risk of developing a recurrence (HR:3.11, 95% CI 1.41–6.86; $p = 0.005$) (Table 7). The leukoplakia that was diagnosed in patients who used snuff was not at the same location as where the patient had applied the snuff, and all the patients who consumed snuff used Swedish moist snuff.

A clinical diagnosis of non-homogenous leukoplakia showed a trend to association with recurrence of leukoplakia ($p = 0.052$), and use of snuff continued to be a significant risk factor for recurrence of leukoplakia after surgery ($p = 0.014$) (Table 7).

Table 6. Clinical factors and treatment outcome for patients with leukoplakia treated with surgical excision (90).

	No recurrence N (%)	Recurrence N (%)	Total N	P-value
Patients	60 (58)	43 (42)	103	
Gender				NS
Male	27 (54)	24 (46)	51	
Female	33 (63)	19 (37)	52	
Clinical diagnosis				0.021
Homogeneous	42 (68)	20 (32)	62	
Non-homogeneous	18 (44)	23 (56)	41	
Size				NS
>200 mm^2	17 (50)	17 (50)	34	
<200 mm^2	43 (62)	26 (38)	69	
Site of lesion				NS
Tongue	18 (50)	18 (50)	36	
Attached gingiva and hard palate	29 (60)	19 (40)	48	
Buccal mucosa and floor of the	13 (68)	6 (32)	19	
Number of lesions				NS
Multiple	27 (53)	24 (47)	51	
Single	33 (63)	19 (37)	52	
Dysplasia				NS
Yes	10 (45)	12 (55)	22	
No	50 (62)	31 (38)	81	
Smoker				NS
Yes	14 (74)	5 (26)	19	
No	46 (55)	38 (45)	84	
Past smoker				NS
Yes	18 (47)	20 (53)	38	
No	25 (60)	17 (40)	42	
ND	3 (75)	1 (25)	4	
Snuff use				0.003
Yes	3 (27)	8 (73)	11	
No	57 (62)	35 (38)	92	
Past snuff use				NS
Yes	11 (79)	3 (21)	14	
No	45 (59)	31 (41)	76	
ND	4 (31)	9 (69)	13	
Alcohol consumption				NS
Low to moderate	51 (61)	33 (39)	84	
Excessive use of alcohol	4 (40)	6 (60)	10	
ND	5 (56)	4 (44)	9	

Recurrence of leukoplakia and malignant transformation

Four of the 43 patients with leukoplakia that recurred developed OSCC at the site of the primary lesion. None of the 60 patients with non-recurring leukoplakia transformed into OSCC, which resulted in a significant difference between the two groups ($p < 0.001$).

Table 7. Cox regression analysis of risk factors for the recurrence of leukoplakia, described as a Hazard Ratio with 95% confidence interval (90).

	Uni-variable analysis		**Multi-variable analysis**	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
Clinical diagnosis				
Homogeneous	1.00		1.00	
Non-homogeneous	2.00 (1.10–3.65)	0.024	1.83 (1.00-3.37)	0.052
Size				
<200 mm^2	1.00			
≥200 mm^2	1.27 (0.69–2.35)	0.45		
Dysplasia				
Yes	1.00			
No	1.60 (0.82–3.13)	0.17		
Number of lesions				
Multiple	1.00			
Single	1.18 (0.65–2.16)	0.59		
Smoker				
Yes	1.63 (0.64–4.15)	0.30		
No	1.00			
Snuff use				
Yes	3.11 (1.41–6.86)	0.005	2.73 (1.22-6.08)	0.014
No	1.00		1.00	

4.4 STUDY IV

Does the expression of the cell proliferation biomarkers p53, p63, podoplanin and Ki-67 correlate with recurrence of leukoplakia?

Study IV shows that the overexpression of p63 is significant associated with an increased risk for recurrence after surgical excision.

5 GENERAL DISCUSSION

Study I: is there a correlation between the over-expression of the tumour suppressor protein p16 and high-risk HPV infection in cases of leukoplakia and oral squamous cell carcinoma?

We show that over-expression of the tumour suppressor protein p16 does not correlate with any of the assessed HPV types, since p16 was over-expressed in several leukoplakias and none of the analysed leukoplakias were positive for any of the 14 analysed HPV types. This finding is surprising because several previous studies have reported higher prevalence rates of HPV in leukoplakia and in OSCC (91-93). In a systematic review, a significant correlation between HPV, OSCC and leukoplakia was reported (20).

As 18% of the patients with leukoplakia over-expressed p16 and none of them was positive for the investigated HPV types, we conclude that over-expression of p16 is not a reliable biomarker of HPV infection in the oral mucosa. Our findings are supported by those of other studies showing that the over-expression of p16 do not correlates with infection by HR-HPV (52-54). However, other studies contradict our findings and maintain the view that p16 is a reliable biomarker of HPV infection in the oral mucosa (49-51).

When we analysed the p16-positive OSCC specimens, 38% of the samples where positive for the investigated HPV types. This is in line with the results of several studies from different geographical regions and with different patient cohorts (35). However, the patient cohort in the present study is not representative of OSCC cases, since the inclusion criterion was tumour over-expression of p16.

When p16 expression was analysed with regard to the presence of epithelial dysplasia, p16 was expressed at a higher level in leukoplakia with dysplasia than in leukoplakia without dysplasia, although no significant correlation was observed. Our results are in line with those presented by Angiero and colleagues, who have suggested that the expression of p16 increases with the degree of dysplasia (94). Assessing the level of p16 over-expression and correlating it to the degree of dysplasia resulted in a sensitivity of 29% and a specificity of 84% for the detection of dysplasia in leukoplakia. These percentages differ from those reported by Pathak and co-workers, who observed 83% sensitivity and 40% specificity (51). Our study and the study by Pathak et. al. shows that p16 is not a reliable biomarker for the detection of dysplasia or for the detection a HPV infection in the oral mucosa.

The tumour suppressor protein p16 acts as part of the internal control of the cell cycle by interfering with the retinoblastoma pathway, preventing the cell from entering the S-phase. In leukoplakia, genomic disturbances in the cell cycle-regulatory mechanisms have been demonstrated, and p16 has been proposed as a prognostic marker for malignant transformation (17).

Thus, it is not surprising that over-expression of p16 is seen in our leukoplakia samples. However, our finding that none of the leukoplakias was positive for any of the investigated HPV types is unexpected. We hypothesised that we would find HR-HPV DNA in the leukoplakias as several studies have addressed the correlation between HR-HPV infection and leukoplakias (20, 36).

Virus harboured in human tissue is by definition an infection. The prevalence rates of infectious diseases often show global variation, and this is the case also for HPV infections. The prevalence of HR-HPV infection of, for example, the cervix uteri shows considerable variation around the world (31).

Following an alarming increase in the number of cases of HPV-positive oropharyngeal squamous carcinomas (OPSCC) in Sweden (38, 39), we wanted to find out if the incidence of HPV-positive leukoplakias has increased over time. We also wanted to know if the prevalence rates of HR-HPV-positive leukoplakias differ between Sweden, Brazil and Romania.

Since we did not detect DNA for any of the investigated HPV types in the leukoplakia cases, we proceeded with investigating the correlation between HR-HPV infection and leukoplakia in a global perspective. In the subsequent study, we used a Real-Time PCR assay instead of the detection of p16 as a surrogate marker for the detection of a HR-HPV infection in the leukoplakias. The study was expanded to geographical regions in South America (Brazil) and central Europe (Romania). Several studies have reported geographical differences in HPV prevalence around the globe (35, 36, 95).

Study II: Has the prevalence of high-risk HPV in leukoplakia changed over time? Does the prevalence differ between Sweden, Romania and Brazil?

Study II shows a surprising low prevalence of HPV infection in 418 analysed leukoplakia cases from the three different countries regarding the 12 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) and two of the low-risk HPV types (6 and 11) analysed in the study. None of the patients with leukoplakia in the European cohorts was positive for the investigated HPV

types, whereas HPV DNA was detected in 3% (5/189) of the leukoplakia samples from the Brazilian cohorts.

Neither the mean nor the median ages of the patients differed between the three countries. The gender distribution was balanced between the Swedish and the Brazilian cohorts but tended towards a majority of females in the Romanian cohorts. Therefore, it seems unlikely that the difference in HPV-positivity between Sweden and Brazil is due to gender or age differences, since the two countries display similar cohort demographics.

In the Brazilian cohorts, there was a higher incidence of dysplastic lesions than in the Swedish and Romanian cohorts. This may explain the higher HPV prevalence in the Brazilian cohorts compared to the European cohorts. However, the correlation between HR-HPV infection and the presence of epithelial dysplasia in leukoplakia is debated. A meta-analysis conducted by de la Cour and colleagues did not show a significant correlation between epithelial dysplasia in leukoplakias and HPV infection (36). A similar meta-analysis carried out by Syrjänen and co-workers showed that the prevalence of HPV infection was higher in dysplastic lesions than in non-dysplastic lesions (20).

When two different time periods, 1992–2002 and 2011–2017, were compared, there were no increase in HR-HPV prevalence between these periods. However, the incidence of HR-HPV infection was too low to draw definite conclusions. In the Brazilian historical cohort, 1.1% of the leukoplakia samples were positive for HR-HPV DNA, and the HR-HPV prevalence was 4.1% in the contemporary Brazilian cohort. Combining the patients from the different countries into a historical and contemporary cohort, only 0.5% of the leukoplakia in the historical cohort were positive for the assessed HPV types, as were 2% of the leukoplakia in the contemporary cohort. These finding are not in line with the observed increase in HPV prevalence in oropharyngeal carcinomas (38-40).

No major difference in HR-HPV prevalence between the three different countries was observed. The results from the Brazilian cohorts indicate a higher prevalence of HR-HPV infection. This may reflect a higher HPV prevalence in the South American population, as has been shown in recently published meta-analyses (36, 96).

Another surprising finding was that 17 patients with leukoplakia that transformed into OSCC did not show the presence of any of the investigated HPV types and the same was true for the corresponding OSCC in these

patients. All these patients were followed from the time-point of leukoplakia diagnosis to the time-point of OSCC diagnosis. This finding is not in agreement with other studies that have demonstrated higher HPV prevalence rates in both leukoplakia and in OSCC cases (35, 36, 96). In another study, HR-HPV types were detected in 10.6% of OSCC in patients in the US, as compared with 6.1% in European patients, and 0% in Brazilian patients (95). However, the results from the second study correspond with those of several other reports, demonstrating that none of the investigated leukoplakias were positive for HR-HPV infection (42, 97, 98). In the previously mentioned meta-analysis by de la Cour et al (36), they addressed the prevalence of HPV in potentially malignant disorders. This meta-analysis included 28 studies that specifically addressed HPV prevalence in leukoplakias. Eight of these studies reported no presence of HR-HPV in cases of leukoplakia in Thailand, China, Iran, India and Sweden (36).

Among the 17 cases of leukoplakia that transformed into OSCC, the majority of the patients were female (16/17 patients). This supports previous findings recognizing the female gender as a risk factor for malignant transformation of leukoplakia (14). Another explanation for the higher number of female patients among the cases of leukoplakia that transformed into OSCC could be the origin of the patients. Seven out of seventeen leukoplakias that transformed to OSCC originate from the Romanian cohorts, where the majority of the patients are of female gender.

The low HR-HPV prevalence in cases of leukoplakia and OSCC is surprising because many studies have reported higher HPV prevalence rates in patients with leukoplakia, other potentially malignant disorders, and OSCC. Pierangeli and co-workers reported that 53.2% of patients with potentially malignant disorders were HPV-positive and that HPV types 16 and 18 were the most frequently detected types (91).

A correlation between leukoplakia and infection with a HR-HPV has been suggested by several groups (20, 43, 92). At the same time, in agreement with our findings, several studies have reported the absence or very low prevalence of HR-HPV in leukoplakias (97, 99, 100). However, there was an expectation to find more HR-HPV-positive leukoplakias in our cohorts, since the HPV prevalence in the healthy oral mucosa is reported to be 4.5%, including a 3.5% prevalence for HPV-16 (101).

That the detection of HPV differs between different studies is an established fact. There are many reasons for this, including the choices of sampling methods and detection techniques. In the present studies, Real-Time PCR was

chosen to detect HPV DNA, for several reasons. A major reason is that the PCR assay has a high sensitivity to detect HPV DNA. However, PCR may also detects an HPV infections that are not transcriptionally active. To be able to detect an active HR-HPV infection, the PCR must detect the transcriptionally active E6/E7 HPV mRNA. Since no HPV-positive samples were detected in the Swedish or Romanian cohorts and in only a few patients in the Brazilian cohort, we decided not to proceed with mRNA detection. Bhosale et al. (42) attempted to assess the presence of HPV E6/E7 mRNA in leukoplakia and in head and neck carcinomas. However, they did not find any mRNA in the cases of leukoplakia since all the samples were negative for HPV infection. In contrast, transcriptionally active HPV was found in 8/427 patients with head and neck squamous cell carcinoma; in these eight patients the tumours were located in the larynx or oropharynx and none had tumours in the oral cavity (42).

The PCR assay used in Studies I and II has high sensitivity and specificity for finding HPV DNA in samples (89). In the assay, an internal positive control with a control plasmid containing target segments from all the investigated HPV types was used. As additional positive controls, previously confirmed HR-HPV-positive OSCC and cervical carcinomas were used. Consequently, the presence of the assessed HPV types in the analysed tissue specimens was highly unlikely (89)

Another possible explanation for the discrepancy between the results from the present studies and the results obtained by other groups is differences in the prevalence rates between geographic regions. In several studies that have analysed HPV prevalence rates in patients with leukoplakia, OSCC or oropharyngeal carcinomas, the global distribution of HPV infection has been addressed, and the conclusion is that there is a scattered distribution of HPV types around the globe (102, 103). This difference was taken into consideration when Study II was conducted since research sites from three different countries were selected.

Another potential factor for the low HPV prevalence rates is HPV vaccination. During the last decades, ongoing community-based vaccination programmes have been conducted in Europe and Brazil. However, the target population for the programme is adolescents, and the patients in our study cohorts were significantly older and have not been a part of the HPV vaccination programme**.** Consequently, the vaccination program cannot be an explanation for the low HPV prevalence seen in our study. We are now in the post-vaccination era and the effects of the different HPV vaccination programmes across the world will soon reveal their potential to prevent cervical carcinomas.

A recent nationwide registry study, assessing 1,672,983 females in the age range of 10–30 years in Sweden, has concluded that HPV vaccination is associated with a substantially reduced risk of cancer of the cervix uteri. The incidence rate is lower in girls who received HPV vaccination before the age of 17 years (104).

Regardless of our finding of low HPV prevalence in our patients with leukoplakia, it is important not to exclude the possibility that HPV belonging to other HPV types (or to genera other than the ones investigated in our studies) may play important roles in leukoplakia and OSCC. The HPV types investigated in our study belong to the α-papillomavirus genus. A study by Viarisio and colleagues showed in a transgenic mouse model that HPV 49 (included in the β-papillomavirus genus) increases the risk for mice to acquire cancer of the upper digestive tract (105, 106). This emphasises the importance of addressing HPV types other than the most frequently investigated ones.

HR-HPV infection may have a causative role in OSCC, albeit not to the same extent as in OPSCC. One explanation for this could be the differences in tissue architecture between the oral cavity and oropharynx. Another reason could be that the immune responses differ between the two different anatomical sites.

The results of Studies I and II indicate that HPV is neither associated with leukoplakias nor linked to the malignant transformation of leukoplakia. This does not mean that an infection with HR-HPV does not lead to OSCC. One hypothesis is that OSCC that is driven by HPV infection is a different type, in that it differs from OSCC preceded by leukoplakia. Therefore, we propose that the correlation between leukoplakia, OSCC and HPV infection needs to be further clarified. The results from Studies I and II indicate that additional larger, prospective, population-based, studies with subjects from different geographical regions and with a wider search for more HPV types need to be conducted. This is so as to clarify the temporal relationship between HPV infection, leukoplakia and OSCC, and to elucidate further the action of HPV as a co-factor in malignant transformation.

Since our results did not indicate that HPV is associated with leukoplakia and malignant transformation of leukoplakia, we decided to proceed with a different approach. Following a review of the ORA-LEU-CAN study, we learnt that many leukoplakia recurred after surgical excision. Recurrence after surgical excision is a clinical problem and it may be a predictor of malignant transformation. This prompted us to investigate this aspect further.

Study III: which clinical and anamnestic factors correlate with the recurrence of leukoplakia after surgical removal?

The main finding of Study III was that, despite total excision of the lesion, a high proportion (42%) of leukoplakias recur.

This is in line with the results of a previous study by Brouns et al. (10), who reported a recurrence rate of 42%. Study III reveals that the cumulative incidence of recurrence after 4 years was 45% (95% CI 35%–56%) and after 5 years it was 49% (95% CI 28%–60%).

Our leukoplakia recurrence rates were considerably higher than those reported from studies that used laser surgery instead of the conventional scalpel removal of the leukoplakias. Regarding laser removal of leukoplakia lesions, Del Corso and colleagues have reported a recurrence rate of 28.5% (107), and Kuribayashi et al. have reported a recurrence rate of 15.1% (108). It is possible that the use of laser surgery influenced the outcomes in the previously mentioned studies.

A recent meta-analysis conducted by de Pauli Paglioni et al. (109), analysing the recurrence rate of leukoplakias after surgical excision treated by laser, demonstrated a recurrence rate of 16.5%. When they compared this with traditional scalpel surgery they concluded that laser surgery had the lowest recurrence rate (109). However, our recurrence rate was higher than that reported by Holmstrup et al. in 2006 (11). Of the 94 patients in their study, which included cases of erythroplakia, 13% recurred after surgical excision. Excluding erythroplakia, only 10/85 patients with leukoplakias recurred (11).

The main outcome of Study III is that a clinical diagnosis of non-homogeneous leukoplakia and the use of snuff are the only clinical factors to predict recurrence of leukoplakia after surgery. In contrast, clinical factors such as the location of the lesion, presence of epithelial dysplasia, gender, size, smoking, use of alcohol, and single or multiple lesions do not show significant differences between recurring leukoplakia and non-recurring leukoplakia. Our results revealing that the clinical diagnosis of non-homogeneous leukoplakia as a risk factor for recurrence after surgical excision is in line with the findings from several previous studies (107, 110).

Of the patients with recurring leukoplakia, 9% suffered a malignant transformation. All the patients with leukoplakia that transformed into OSCC experienced a recurrence of the leukoplakia before malignant transformation. This resulted in a significant difference between the two groups. These

findings strengthen the assertion that even when there is surgical removal of the lesion the risk of malignant transformation is not eliminated (10, 11). The recurrence of leukoplakia after surgical excision also indicates that in the periphery and clinically healthy tissue surrounding the lesion, areas that are not included in the excision margin, there exists genomic instability and disturbance of the cell-cycle-regulatory mechanisms (111). Stringent regulation of the cell cycle and cell division is essential for avoiding malignant transformation (6).

An important concept in understanding the malignant transformation of leukoplakia is `field cancerization´. This is accepted as an explanation for the recurrence after surgical excision and malignant transformation of leukoplakia (111, 112). The concept implies that genomic instability is generalised in the mucosa surrounding the lesion. This genomic instability entails an increased risk of genetic aberrations occurring in keratinocytes through random mutations, which may lead to OSCC. The concept of field cancerization can be applied to leukoplakia, since there is genomic instability in the epithelium (17).

There is an urgent need for robust biomarkers that will facilitate risk assessment of leukoplakia. Genetic aberrations causing disturbances of cell cycle control are key factors in cancer development (7). The role in leukoplakia of molecules involved in cell cycle control has been extensively addressed previous studies (17). Therefore, we hypothesise that there is a difference in cell proliferative signalling in epithelial cells in recurring versus non-recurring leukoplakia.

Study IV focused on biomarkers related to cell cycle control and the correlation between these markers and recurrence of leukoplakias after surgical excision. This is based on the results from study III, in which we proved that recurrence is a risk factor for malignant transformation. Thus, we wanted to identify biomarkers that may predict the recurrence of leukoplakia after surgical removal, so as to facilitate the cancer risk assessment.

Study IV: Can expression of the cell proliferation biomarkers p53, p63, podoplanin and Ki-67 predict the recurrence of leukoplakia after surgical excision?

The main finding of Study IV is that the expression of p63 is associated with an increased risk of recurrence of leukoplakia. In addition, the levels of Ki-67 showed a tendency towards significant differences between leukoplakias that did recur and those that did not recur.

We showed in Study III that recurrence after surgery was an important risk factor for malignant transformation of leukoplakia. The fact that leukoplakia recurs after complete surgical removal is an indication of disruption of the cell cycle control. Two of the four biomarkers examined in Study IV, i.e., p53 and p63, are associated with control of the cell cycle and cancer development (7).

A recent meta-analysis performed by Mello and co-workers summarised studies that have proposed different combination of biomarkers for predicting malignant transformation (66). Nevertheless, studies addressing biomarkers related to the recurrence of leukoplakia are sparse. A subsequent step would be to identify a set of biomarkers that predicts recurrence and combine this with clinical parameters so as to create an algorithm that could guide the clinician in the risk assessment of the leukoplakia and with decisions as to the treatment and follow-up interval.

5.1 METHODOLOGICAL CONSIDERATIONS

A limitation of the ORA-LEU-CAN study is that the patient cohort is not representative of the Swedish general population. In the ORA-LEU-CAN study, all the patients are referred from general dental and medical practitioners and are under treatment in routine dental care. High-risk patients do not attend routine dental treatment appointments to the same extent as the patients in the ORA-LEU-CAN study. Previous studies have shown that both leukoplakia and OSCC are more common in patients who are heavy consumers of alcohol and tobacco (111), a patient group that is not represented in the ORA-LEU-CAN study.

Another limitation is that Study II has a partly retrospective design. This raises the issue of the reliability of the leukoplakia diagnosis made for these patients. Since leukoplakia is an exclusion diagnosis, there is a risk that other disorders in the oral mucosa that resemble leukoplakia are included in the study. All the patients were examined, diagnosed and biopsied by specialists in oral maxillofacial surgery or oral medicine, which reduces this risk, although it cannot be completely eliminated.

The fact that the ORA-LEU-CAN study is a multi-centre study may introduce a diagnostic bias related to differences in the interpretations of the inclusion criteria and definitions of leukoplakia across the sites. In a clinical exclusion diagnosis, there will always be a risk of inter- and intra-observational variabilities, leading to a variation in the leukoplakia diagnosis. That risk was reduced by allowing two specialists in oral medicine to re-review the clinical diagnosis. Since the diagnosis of leukoplakia has gone through several assessments, we can state with confidence that the leukoplakia diagnosis is correct and safe. Likewise, we can be confident of the diagnosis of the recurrence of leukoplakia after surgical excision, since the finding of a clinical healthy mucosa had to be supported in the medical records with a clinical photograph (Figure 6).

Furthermore, a strength of this thesis is the patient material. The fact that the ORA-LEU-CAN study has a prospective design and all the patients are well-documented strengthens the validity of the subsequent studies.

In the ORA-LEU-CAN study, seven patients with the clinical diagnosis of leukoplakia had received the histopathological diagnosis of lichenoid reaction. In these patients, the clinical re-review was extra thorough, and the medical records and clinical images were re-reviewed intensively. No clinical signs, according to the definitions of van der Meij et al (112), were found to indicate

that the lesion was an oral lichenoid lesion, oral lichen planus or lichenoid contact reaction. Leukoplakia is a clinical diagnosis and we strictly adhered to the WHO definition of leukoplakia, which postulates exclusion of known diseases or disorders that carry no increased risk of developing cancers and in which the histopathological diagnosis is restricted to detecting the presence of dysplasia or not (1). Consequently, when excluding all other clinical diagnoses that might evidence a white plaque in the oral cavity the histopathological diagnosis of lichenoid reaction still fits the clinical diagnosis of leukoplakia.

Determining the margins of leukoplakias during surgery remains a challenge. According to the study of Kuribayashi et al., there is a significant correlation between extension of the surgical margins and the recurrence of leukoplakia after excision. (108). To ensure that the leukoplakia is fully excised, serial sectioning of the biopsy specimens and histopathological assessment are needed. We did not perform these analyses, since the ORA-LEU-CAN study is a clinical study and serial sectioning is not part of the clinical routine. However, experienced specialists in maxillofacial surgery and in oral medicine performed the surgery and the clinical evaluation of the surgical margins, which reduces the risk that any remnants of the leukoplakia were left during the surgery. To reduce the risk that a leukoplakia is not fully excised and, consequently, cannot be considered as a recurrence, a clinically healthy mucosa had to be verified in the medical journals with a photograph at the follow-up visit. This was later followed by a re-review of a photograph from the medical record showing a new leukoplakia at the same site.

5.2 THE RATIONALE BEHIND THE STUDIES IN THIS THESIS

Currently, the correlations between p16 and HPV infection in leukoplakia and OSCC are debated and uncertain. The aim in Study I was to explore the correlation between p16 over-expression and HPV infection in both leukoplakia and OSCC. Using the findings from Study I and knowledge from previous HPV prevalence studies, we designed Study II to determine: 1) whether the incidence of HPV-positive leukoplakia has change over time; and 2) if the prevalence rates of HPV-positive leukoplakia differ between Sweden, Brazil and Romania.

Based on the findings obtained in Study II, we decided to adopt a different approach: to analyse the recurrence of leukoplakia after surgical excision, which has been shown to be a predictor of malignant transformation of leukoplakia and is a problem in the clinical setting.

In the two subsequent studies (III and IV) we characterised the clinical, anamnestic and molecular factors with potential to predict recurrence of leukoplakia after surgical removal. Thus, the aim of prospective Study III was to uncover the clinical and anamnestic factors that correlate with recurrence of leukoplakia. In Study IV, we investigated whether the expression of four biomarkers correlates with the recurrence of leukoplakia. We hypothesised that there is a difference in the cell-proliferative signalling in epithelial cells between recurring leukoplakia and non-recurring leukoplakia.

6 CONCLUSIONS

6.1 STUDY I

- Over-expression of p16 is not a reliable biomarker for the detection of a HR-HPV infection in patients with leukoplakia or OSCC.

6.2 STUDY II

- There has been no clear increase over time in the prevalence of HR-HPV infection in leukoplakia in Sweden, Romania and Brazil.
- Brazilian patients displayed a low prevalence of HR-HPV infection in leukoplakia, while HR-HPV was not detected in leukoplakias from patients in Swedish and Romanian.
- The assessed HPV types could not be detected in cases of leukoplakia that preceded OSCC, neither in the corresponding OSCC.
- Larger, prospective and population-based, multi-national studies are required to explore the prevalence of HR-HPV infection and outcomes for patients with leukoplakia.

6.3 STUDY III

- The cumulative incidence of recurrence of leukoplakia is found to be 45% after 4 years and 49% after 5 years.
- Clinical parameters that predict the recurrence of leukoplakia after surgical removal are the non-homogeneous clinical type and the use of snuff.
- Leukoplakia that recurs has a significantly higher risk of transforming into OSCC.

6.4 STUDY IV

- A high expression of tumour suppressor proteins p63 was associated with increased risk for recurrence of leukoplakia after surgical excision.

7 FUTURE PERSPECTIVES

Oral leukoplakia still remains an enigma. The potential for the disorder to develop into squamous cell carcinoma poses a challenge for clinicians and scientists around the globe. Treatment, follow-up and early identification of cancer warrant robust decision algorithms. Thus, the quest for reliable clinical features and stable biomarkers predicting cancer transformation are important research areas.

In the present research projects the role of human papillomavirus infection in leukoplakia has been investigated. HR-HPV is a well-known initiator of cancer in cervix uteri and has been attributed a role in cancer development in the oral cavity. The results from the first study show that tumour suppressor protein p16 – a well-established HPV surrogate marker in oropharyngeal carcinoma- cannot be used as a surrogate biomarker for HPV infection in leukoplakia or oral squamous cell carcinoma. To establish HPV infection in leukoplakia fast and simple techniques for detecting types of HPV are needed.

Several studies have reported that HPV infection in leukoplakia shows a global variation. The second study in this thesis could not present a large variation between Sweden, Romania and Brazil. Nor was an increase in HPV infection rates detected for the investigated types. However, this does not exclude HPV infection with other types than the types assessed in this study why broader panels of HPV types should be used in future studies. The increased incidence of HPV-infected oropharyngeal carcinomas over the last decades open questions whether this may become true also for oral leukoplakia and oral cancer. This highlights future possibilities for screening of patients with potentially malignant oral disorders.

Surgical treatment of leukoplakia has in earlier studies not been shown to be especially successful. In the studies presented in this thesis further lends support to the need for developing new surgical techniques for leukoplakia treatment. Patients with leukoplakia that recurred after surgical excision developed oral cancer to a higher extent than patients without signs of recurrence. Thus, recurrence *per se* is a risk factor for cancer. The question whether of surgery increases cancer risk has been proposed. The creation of a surgical wound leads eventually lead to a healing process. In the healing process a tight control of cell proliferation is important, especially in tissue where cells may have genetic aberrations influencing cell cycle regulation. Future diagnostic models may include non-invasive techniques such as *in vivo* spectroscopy to determine surgical margins.

The last part of this thesis explored biomarkers related to cell cycle regulation. The tumour suppressor protein p63 was found to predict recurrence after surgery. The other investigated molecules- p53, Ki-67 and podoplanin- did not turn out predictive. But future research should focus on exploring combination of biomarkers involved in cell proliferation. Combining biomarkers involved in governing cell faith can give tools to risk stratify patients with leukoplakia. Since uncontrolled cell proliferation is one of the cancer hallmarks, biomarkers identifying aberrant control of cell division in keratinocytes in healthy oral epithelium or in leukoplakia may be an avenue to construct decision algorithms for treatment and follow-up schemes.

In summary, the findings in this thesis may open avenues for future research and to answer the question which leukoplakia that will become cancer.

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APPENDIX

I. **Sundberg J**, Korytowska M, Miranda Burggos P, Blomgren J, Blomstrand L, De Lara S, Sand L, Hirsch JM, Holmberg E, Giglio D, Öhman J, Kovács A, Horal P, Lindh M, Kjeller G, Hasséus B. Combined testing of p16 tumour-suppressor protein and human papillomavirus in patients with oral leukoplakia and oral squamous cell carcinoma. *Anticancer Research* 2019; 39: 1293-1300.

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