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Knežević, Marjana

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UNIVERSITY OF ZAGREB
SCHOOL OF DENTAL MEDICINE

Marjana Knežević

**THE ROLE OF HUMAN PAPILLOMAVIRUS
(HPV) IN THE DEVELOPMENT OF BENIGN
AND MALIGNANT CHANGES OF THE
ORAL MUCOSA**

DOCTORAL THESIS

Zagreb, 2016



SVEUČILIŠTE U ZAGREBU
STOMATOLOŠKI FAKULTET

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**ULOGA HUMANIH PAPILOMA VIRUSA
(HPV) U RAZVOJU DOBROĆUDNIH I
ZLOĆUDNIH PROMJENA SLUZNICE
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Mentor: Professor Marinka Mravak-Stipetić, DMD, MSc, PhD
Co-mentor: Magdalena Grce, PhD

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Komentor: Dr.sc. Magdalena Grce

Zagreb, 2016.

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Title of Postgraduate Doctoral study: Dental medicine

Mentor: Professor Marinka Mravak-Stipetić, DMD, MSc, PhD, Department of Oral medicine, School of Dental Medicine, University of Zagreb, Croatia

Co-mentor: Dr Magdalena Grce, PhD, Rudjer Bošković Institute, Zagreb, Croatia

Editor for English and Croatian language: Marina Lončar, professor of English and Croatian language and literature, librarian.

Commission for evaluation of doctoral Thesis:

1. Assoc. Prof. Vlaho Brailo, president, School of Dental medicine University of Zagreb, Croatia
2. Professor Irina Filipović Zore, member, School of Dental medicine, University of Zagreb, Croatia
3. Assoc. Prof. Romana Čeović, member, School of Medicine, University of Zagreb, Croatia
4. Assis. Prof. Krunoslav Kuna, member, University Hospital Clinic Sisters of Mercy, Zagreb, Croatia
5. Professor Ivan Alajbeg, member, School of Dental medicine, University of Zagreb, Croatia

Commission for doctoral Thesis defence:

1. Assoc. Prof. Vlaho Brailo, president, School of Dental medicine University of Zagreb, Croatia
2. Professor Irina Filipović Zore, member, School of Dental medicine, University of Zagreb, Croatia
3. Assoc. Prof. Romana Čeović, member, School of Medicine, University of Zagreb, Croatia
4. Assis. Prof. Krunoslav Kuna, member, University Hospital Clinic Sisters of Mercy, Zagreb, Croatia
5. Professor Ivan Alajbeg, member, School of Dental medicine, University of Zagreb, Croatia

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SUMMARY

Objectives: The aim of this study was to determine the prevalence and type of human papillomavirus (HPV) in various oral lesions in comparison to control healthy oral mucosa, and the dynamic of HPV infection course (persistence, change of HPV type and clearance).

Material and Methods: The study comprised 426 consented subjects, of which 343 patients with different oral lesions and 83 controls with apparently healthy oral mucosa, referred for oral examination in the period from 2000 to 2015. The diagnoses of oral lesions were established according to clinical criteria and confirmed by histopathology. Oral cytobrush samples were collected from oral lesions and healthy mucosa from different topographic sites and the HPV types were determined by polymerase chain reaction (PCR). The presence of HPV DNA was evaluated by consensus and type-specific (TS) primer-directed PCR. A subset of samples was analysed for the presence of both alpha and beta genus HPV types.

Results: Out of 426 specimens, 144 (41.98%) from oral lesions and 15 (18.07%) controls were positive for HPV DNA; 22.3% of all tested samples contained beta-HPV type and 15.02% alpha-PV type. The highest prevalence of beta-HPV and high risk (HR) HPV (alpha-HPV) types were found in benign and premalignant oral lesions, while HR-HPVs were found in a small number of control samples. In 38 subjects sampling was performed more than twice, in average 2.29 times, with time distance between sampling of 5.8 months. Twenty-six of these 38 patients were continuously HPV negative, five had transient infection, five acquired a new HPV infection, one had persistent HPV infection, and one had initially co-infection with different HPV types followed with new one during which initial oral lesion became malignant. The topographic distribution of the alpha-PV and beta-PV types showed affinity for the anterior part of the oral cavity and particular risk localization.

Conclusion: Positive finding of HPV in all age groups and on healthy oral mucosa, high prevalence of HPV types in oral premalignant and proliferative lesions, affinity for risk localization in the oral cavity, unpredictable dynamic course and change of HPV type indicates the need for regular oral assessment and HPV control in patients with initially positive findings on oral mucosa.

Keywords: Oral cavity, benign oral lesions, premalignant oral disorders, oral cancer, human papillomaviruses (HPV), alpha-PV type, beta-PV type

SAŽETAK**Uloga humanih papiloma virusa (HPV) u razvoju dobroćudnih i zloćudnih promjena sluznice usne šupljine**

Cilj istraživanja bio je ispitati prevalenciju i tip humanog papiloma virusa (HPV) u različitim oralnim lezijama u usporedbi s kontrolnom, naizgled zdravom sluznicom i dinamiku tijekom HPV infekcije u ustima (ustrajnost, promjenu tipa HPV i prolaznost infekcije).

Ispitanici i postupci: U ispitivanju je sudjelovalo 426 ispitanika, od kojih 343 bolesnika s različitim oralnim lezijama i 83 kontrolnih s naizgled zdravom sluznicom koji su upućeni na pregled usne šupljine u razdoblju od 2000. do 2015. godine. Svi ispitanici dali su svoj informirani pristanak prije uključivanja u ovo istraživanje. Dijagnoze oralnih lezija postavljene su na temelju kliničkih kriterija i potvrđene histopatološkom analizom. Citološki obrisci uzeti su citološkom četkicom s oralnih lezija i zdrave sluznice s topografski različitih mjesta sluznice, a HPV je dokazan lančanom reakcijom polimerazom (PCR). Prisutnost HPV DNA dokazana je PCR-om sa zajedničkim i tip-specifičnim početnicama. Određeni dio uzoraka bio je testiran na tipove HPV-a iz roda alfa i beta.

Rezultati: U svih 426 testiranih uzoraka, HPV DNA dokazana je u 144 (41,98%) uzoraka s oralnih lezija i 15 (18,07%) kontrolnih; 22,3% ispitanih uzoraka sadržavalo je tip HPV iz roda beta, a 15,02% tip HPV iz roda alfa. Najveća prevalencija beta-HPV i visokorizičnih (HR) HPV tipova (alfa-HPV) pronađeni su u benignim i premalignim oralnim lezijama, a visokorizični HPV tipovi pronađeni su i u malom broju kontrolnih uzoraka. U 38 ispitanika uzorkovanje je provedeno više od dva puta, u prosjeku 2.29 puta, u vremenskom razmaku od prosječno 5,8 mjeseci. Dvadesetšest od 38 ispitanika bilo je stalno negativno na HPV, u pet je dokazana prolazna infekcija, pet je steklo novu infekciju HPV-om, jedan ispitanik imao je stalnu infekciju HPV-om, a jedan je tijekom praćenja početno dokazanu koinfekciju s različitim tipovima HPV-a i stekao novu s HPV tipom visokog rizika tijekom koje je inicijalna premaligna lezija sluznice postala zloćudna. Analiza topografske distribucije alfa-HPV i beta-HPV tipova na oralnoj sluznici pokazala je afinitet virusa za sluznicu prednjeg dijela usne šupljine i osobito za rizične lokalizacije.

Zaključak: Pozitivan nalaz HPV-a u svim dobnim skupinama i na zdravoj sluznici te visoka prevalencija HPV tipova u oralnim premalignim i proliferativnim lezijama kao i afinitet za rizične lokalizacije te nepredvidljiv dinamičan tijek uz promjene tipa HPV-a upućuju na potrebu kontrole na HPV u kliničkoj stomatološkoj praksi kod inicijalno pozitivnog nalaza.

Ključne riječi: usna šupljina, dobroćudne oralne lezije, premaligne lezije, rak usne šupljine, humani papilomavirusi (HPV), alfa-HPV, beta-HPV

LIST OF ABBREVIATIONS

DNA - deoxyribonucleic acid

E6 - early gene of HPV coding for the oncoprotein E6

E7 - early gene of HPV coding for the oncoprotein E7

HLA - human leukocyte antigen

HPV - Human papillomavirus

HR - high risk (HPV type)

HSV - Herpes simplex virus

LCR - long control region

LR - low risk (HPV type)

OLL - oral lichenoid lesions

OLP - oral lichen planus

p53 - tumour suppressor protein 53

PCR - polymerase chain reaction

RB - retinoblastoma protein, a tumour suppressor protein p53

RNA - ribonucleic acid

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1. INTRODUCTION

1.1. Human papillomavirus (HPV)

Human papillomavirus (HPV) has a double-helical DNA genome and is taxonomically classified in a Papillomaviridae family (1). They were first discovered, isolated, and sequenced from cervical tumor specimens and have been attributed to be one of the important causative agents for the development of cervical cancer by zur Hausen et al. (2, 3, 4). For this discovery Professor zur Hausen received the Nobel Prize of Physiology and Medicine for the year 2008.

HPV have strict tropism and infect either mucosa (alpha genus papillomaviruses) or skin (beta and gamma genus papillomaviruses). Genital HPV infections contribute to more than 99% of cases of cancer of the cervix (5), 97% of cancer cases of the anus (6), 70% of cancer cases of the vagina (7), 47% of cancer cases of the penis (8), 40% of cancer cases of the vulva (7) 47% of cancer cases of the oropharynx, and 11% of cancer cases of the oral cavity (9, 5). There are more than 200 HPV genotypes, of which approximately 40 types infect the anogenital and oral mucosa, whereas the others infect the skin (10, 11).

Alpha-HPVs have historically been classified according to their oncogenic potential and clinical behavior into low-risk (LR) HPV types, which were rarely or if ever found in cancer, and high-risk (HR) or carcinogenic types (12, 13) which were often found in cancer. The most common HR HPVs, types 16 and 18, cause more than 70% of cervical cancers and other anogenital carcinomas in women (vulvar, vaginal, and anal carcinomas) and men (penile and anal carcinomas), as well as oropharyngeal tumors in both men and women (14). The more recent classification classifies HPV types as carcinogenic (HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), probably carcinogenic (type 68), and possibly carcinogenic (types 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, 97) (15- 17).

In contrast with the HR types, the LR HPV types, notably types 6 and 11, cause almost all clinically visible benign lesions, that are genital warts (flat condyloma and condyloma acuminata) and laryngeal papilloma (15). Oral condyloma acuminata and oral squamous cell papilloma are mostly caused by alpha-HPV types 6 and 11, whereas oral verruca vulgaris is caused by beta-HPV types 2 and 4 (12). Focal epithelial hyperplasia of the oral mucosa (Heck disease) is more common among Eskimos and American Indians than in most other population groups in the world (18). This disease has nonpapillomatous but rather a papular appearance and does not undergo malignant transformation. Focal epithelial hyperplasia is particularly associated with alpha-HPV types 13 and 32, and the HPV prevalence varies from 7% to 36% (19,20). Laryngeal papillomatosis (also known as recurrent respiratory papillomatosis) is a rare

condition consisting of benign tumours of the larynx or other areas of the respiratory tract, which are caused by LR HPV types 6 and 11 (21). Because the disease is most commonly found in children, disease is probably acquired during vaginal childbirth from the HPV-infected mother. Without treatment, it is potentially fatal, as uncontrolled growths may obstruct the airway (21).

1.2. HPV associated lesions of the oral mucosa

HPV is considered to be associated with oral potentially malignant disorders (OPMDs) such as oral leukoplakia, oral lichen planus, and erythroplakia (22, 23).

Leukoplakia is the most common premalignant oral lesion with dysplasia, present in 5% to 25% of biopsies (24). Studies using polymerase chain reaction (PCR) (25) and genotyping have detected LR, HR, and multiple type HPV infections in oral leukoplakia (26,27). Dysplastic leukoplakias (26.2%) (28) are more likely than normal mucosa (10-13%) (28,29) to be infected with HPV. However, studies showing that oral precancerous lesions have a low viral load and that viral integration seldom occurs (30) strongly suggest that the virus is often there as a passenger (31) having little role in neoplastic transformation of these lesions.

In the study of Szarka et al. (32) HPV was detected more frequently in OPMDs lesions than in controls, and the HPV prevalence rate increased gradually with the severity of the lesions, from 32.8, 40.9 to 47.7% in oral lichen planus, oral leukoplakia, and oral squamous cell carcinoma (OSCC), respectively. Both LR and HR HPV types can be found in potentially malignant oral disorders (33) although only HR HPVs will lead to cancer.

Erythroplakia is a lesion with the highest potential for malignant transformation (34). Nielson et al. (35) found HPV in 50% of the erythroplakias alone, whereas the overall HPV detection rate of all premalignant lesions (erythroplakias, homogenous leukoplakias, nodular and verrucous leukoplakias) including erythroplakias was 40.8%. Although the role of HPV causing cervical cancer is well established yet controversy surrounds regarding their role in oropharyngeal malignant and premalignant lesions and has attracted the researches throughout the world (7, 8, 9, 36).

Infection of cervical epithelium with specific HR HPV types plays a fundamental role in the development of cervical cancer, by causing precursor lesions and maintaining malignant growth (37). Two review articles indicated that a similar mechanism is seen in oral potentially malignant disorders (38, 39). The first evidence of HPV association with OSCC (40) appeared

in 1983, revealing that about 61,500 oropharyngeal cancers, associated with HPV infection, which includes lingual cancer, tonsil cancer, and oropharyngeal cancer, occurred globally (41). These cancers were much more common in men (48,900 cases) than in women (12,600 cases). HPV-positive oropharyngeal cancers are associated with oral sex, age younger than 60 years, infrequent p53 gene mutation, and a more favourable clinical outcome, whereas HPV-negative cancers are associated with smoking, excessive alcohol use, age older than 60 years, frequent p53 gene mutation, and poor prognosis (42). HPV contributes to 47% of oropharyngeal cancers and 11% of oral cancers where HPV 16 was most commonly found in oropharyngeal (90%) and oral (96%) cancers (9).

1.2.1. Oral precancerous lesions and cancer

The clear association between HPV and cervical cancer is not as strong for OSCC. Numerous studies have examined the relationship between HPV and oral squamous cell carcinoma OSCC (38-40). HPV integration into host DNA is less common in OSCC (39, 40). Kreimer et al. (41) found the prevalence of HPV to be 24% while Leemans et al. (42) found 35% in OSCC. Based on the latest review study of Gupta & Gupta (22) that included 50 studies on HPV prevalence in OSCC, oral lichen planus, verrucous carcinoma, oral leukoplakia, and benign and malignant papillary lesions during the period of 1994-2014, the frequency of HPV in OSCC varied from 0% to 80%. The HPV type most commonly detected in OSCC and OPMDs were HPV 16 and 18. HPV type 6 and 11 were mostly found in oral benign lesions and papilloma (43, 44, 45). The possible variation could be attributed to difference in ethnicity, geographic locations to variations in methods used for detection of HPV (46). Oral leukoplakia is the most common premalignant lesion that occurs in the oral cavity (47), although the majority of oral leukoplakia would not develop into malignant tumour. In areas of the world where smokeless-tobacco use is common, there is higher prevalence. Moreover, leukoplakia is more likely to occur with increasing age (48). The rate of transformation of oral leukoplakia into oral squamous cell carcinoma (SCC) varies approximately from 0 to 20%, and this malignant transformation may take 1 to 30 years (49). The role of HPV 16 in the causation of oral leukoplakia is unclear although numerous studies indices a strong independent risk factor for oral leukoplakia (32, 50-57).

1.3. Progression of HPV-associated lesions, HPV persistence and clearance

The normal oral mucosa may act as a reservoir for new HPV infections and/or as a source of recurring HPV-associated lesions. The prevalence of HPV in normal oral mucosa range from 0.6% to 81% (36, 58, 59) whereas HPV is spread by skin-to-skin, skin-to-mucosa, and mucosa-to-mucosa contact. Transmission of HPV infection may occur through sexual contact, autoinfection, and occasionally through perinatal transmission of the neonate during its passage through and infected birth canal of the mother (60). Oral HPV acquisition was found to be more positively associated with number of recent oral sex and open mouth kissing partners than with the number of vaginal sex partners (58, 59).

HPV infections are generally transient, with 60 to 70% of new infections clearing within 1 year and 91% clearing within 2 years (61). Only a small proportion of HPV infections progress to persistent infection, often involving HR HPV types that have been shown to persist longer than

LR HPV types (61). Cervical cancer precursor lesions persist longer and progress more quickly in women with HPV 16 and/or 18 infections than in women with other HR HPV types and LR HPV types (12). HPV 16- and HPV 18-positive women have 200-fold increased risk for cervical cancer (62, 63). Factors that may influence progression include coinfection with other sexually transmitted infections such as *Chlamydia trachomatis*, herpes simplex virus (HSV) or human immunodeficiency virus (HIV), tobacco smoking, high parity (more children) and immune suppression (64). The persistence of HPV infection is a major risk factor for carcinogenesis (65) with in addition chronic inflammation and bacterial coinfections that favor HPV infection (66).

The HPV genome contains several early and late open reading frames coding for replication and transcription regulating proteins, E1, E2, E5, E6, E7 and viral capsid proteins, L1 and L2, respectively. Two early proteins, E6 and E7 act as a major viral oncoproteins, while the E5 acts as an auxiliary oncoprotein (12, 67). Both E6 and E7 are small proteins, approximately 18 and 13 kDa in size, respectively, localized in the nucleus (68). The E6 proteins are also found in the cytoplasm and the E7 probably also has cytoplasmic component (69). The expression of the HR E7 proteins by themselves can immortalize human keratinocytes at a low frequency but E6 has no such activity. The combination of E6 and E7, however, is highly efficient at transforming and immortalizing most types of primary cells (70). In addition to E6 and E7, the E5 oncoprotein plays an auxiliary role in cell transformation. Both HR and LR HPV oncoproteins bind their target proteins but HR do it with higher affinity and in addition often degrade them unlike to LR HPV oncoproteins (71).

The transforming properties of HPV oncoproteins lie in the interaction with numerous host cell proteins resulting in the maintenance and the re-entering into the cell cycle, which consequently allows the virus to replicate as it is dependent on the host cell DNA replication machinery. In addition, the E7 protein, together with the E6 oncoprotein of the HR HPV types, is able to interfere with key cellular processes, such as cell cycle, senescence, differentiation, apoptosis and telomere shortening. Furthermore, because of the frequent integration of the HR HPV genome into a host cell chromosome, those two proteins are the only viral proteins that are consistently expressed in HPV associated cancers (16). The HPV E6 and E7 proteins also associate with enzymes that modulate histone acetylation and thus, broadly regulate the transcriptional competence of host cell chromatin (72).

This study is the continuation of our previous study shown in the master's degree thesis defended in April 2008 and partially published (36).

Herein, we show the findings of the follow-up of our patients including new cases to get a better insight into the dynamics of the course of HPV infection on oral mucosa and its association and influence on the course of particular oral lesions.



2. AIM OF THE STUDY

This study is a continuation of longitudinal research on oral lesions, which began in 1995. The aim of the study was to monitor the prevalence and the natural history of HPV infection in oral cavity associated with oral lesions.

In our preliminary published results (36) we have shown that HPV DNA positive finding in oral cavity is linked predominantly to particular anatomical sites rather than the diagnosis itself indicating that topography plays an important role in HPV prevalence findings in oral mucosa and oral lesions.

Herein, in the period from 2000 to 2015 we continued to follow-up our patients and included new ones in this study, in order to get a better insight into the dynamics of the course of HPV infection on the oral mucosa and its association and influence on the course of particular oral lesions.

Thus, the specific objectives of this study were to determine:

- the prevalence of different HPV types in different oral lesions in the cohort of oral medicine patients in comparison to controls with healthy oral mucosa,
- whether the oral HPV infections are transient or permanent,
- whether the HPV-associated lesions progress to a more severe form of disease,
- whether during the follow-up of HPV infection on oral mucosa, the type of virus changes,
- the distribution of alpha-HPV and beta-HPV types on oral mucosa and its prognostic significance, and finally
- the risk of the oral HPV infection in order to assess the need for regular monitoring for oral cavity.



3. MATERIAL AND METHODS

3.1. Study population

The study comprised 426 subjects of which 343 patients with oral mucosal lesions (test group) and 83 controls with apparently healthy oral mucosa. The test group consisted of 275 women (aged 4-83 years) and 151 males (aged 4-82 years). The average age of all subjects was 46.46 years (Table 1). All included subjects were referred for oral examination to the Department of Oral Medicine, School of Dental Medicine, University of Zagreb and assessment was performed in the period from 2000 to 2015.

As this study builds upon our earlier longitudinal research on the role of HPV in the oral cavity and the observation HPV natural history in association with oral lesions, it included the individuals of our previous report (36). We continued to follow up these patients and included new to 2015, with regard to their clinical diagnoses and HPV presence in order to get insight into the dynamics of the course of HPV infection on oral mucosa and its association with the course of oral lesions.

Inclusion criteria for all patients were:

- their written consent to participate in this research
- the presence of oral lesion suspected to be associated with HPV infection
- oral lesions with increased risk of malignant change
- lack of knowledge of present or previous HPV infection anywhere in the body

Exclusion criteria were:

- that patients were under immunosuppressive therapy at the time of sampling.

3.1.1. Ethic statement

Before the year 2001 verbal patient consent was obtained at the time of sample collection, and since then, written informed consent has been obtained from the participants. All relevant patient data (anamnestic, clinical diagnosis), the DNA extracted from oral specimens and HPV testing results were processed anonymously. HPV testing was done on demand through Laboratory service request forms, which had to be signed and stamped by the practicing oral medicine specialist, and approved by the Ruđer Bošković Institute of Zagreb. The whole study was approved by the Ethical Board, School of Dental Medicine, University of Zagreb in line with the Helsinki declaration.

Consent patients and controls were included into the study in order as they came for clinical examination. In all patients, clinical diagnoses were established based on the medical history, physical examination and routine oral tests.

Clinical diagnosis for oral premalignant lesions were further confirmed by histopathology (23). Repeatedly toluidine positive ulcerous lesions were also subjected to biopsy and histologically analysed.

3.2. Patient samples

From all consent 343 patients with oral lesions and 83 consent controls, oral scraping samples taken by cytobrush (Medscand AB, Sweden) were collected from various topographic sites and labelled according to the scheme WHO as described previously (36). A cytobrush was used for taking scraping at the site of clinically visible lesion, which were classified by clinical diagnosis, and from healthy mucosa in controls as well.

Cytological samples were taken from clinically distinct oral lesions (potentially malignant lesions: 34.27% benign lesions, 23.24% inflammatory lesions 9.86% ulcerations 6.81% salivary gland diseases 4.93% and oral carcinoma 1.41% collected during oral examination at the Department of Oral Medicine, School of Dental Medicine, University of Zagreb. Control samples (n=83) with apparently healthy mucosa were also collected and analysed.

Cytobrush samples from oral mucosa were immediately immersed in an Eppendorf conical tubes filled with 1.5 ml of sterile buffer for extraction (10 mM Tris pH 7.5, 1 mM EDTA pH 7.9, 0.5% SDS) in the presence of proteinase K (100 μ g/ml) in which the cells from mucosal

scraping were lysed. By the time of transport to the laboratory of the Department of Molecular Medicine, RBI, samples were stored in a freezer at -20°C for HPV detection and genotyping.

3.3. Oral topography

The topography of the oral mucosa is coded according to the modified WHO oral topography codes by Roed-Petersen and Roenstrup (Kramer et al 1980; Mattila et al 2012)(36) vermilion border - upper (13), lower (14), labial commissures - right (15), left (16), labial mucosa - upper (17), lower (18), labial sulci - upper (21), lower (22), cheek (buccal mucosa) - right (19), left (20), buccal sulcus - right upper (23) lower (24), buccal sulcus – left upper (25) lower (26), upper gingiva or edentulous alveolar ridge buccally - right (27), left (28), lower gingiva or edentulous alveolar ridge - right (29), left (30), upper anterior gingiva and edentulous ridge labially (31), lower anterior gingiva or edentulous ridge labially (32), upper posterior gingiva or edentulous alveolar ridge palatally - right (33), left (34), lower posterior gingiva or edentulous alveolar ridge lingually - right (35), left (36), anterior gingiva or edentulous ridge palatally (37), and lingually (38), dorsum of the tongue - right (39), left (40), base of the tongue - right (41), left (42), tip of the tongue (43), margin of the tongue - right (44), left (45), surface of the tongue - right (46), left (47), frontal floor of mouth (48), lateral floor of mouth - right (49), left (50), hard palate - right (51), left (52), soft palate - right (53), left (54), anterior tonsillar pillar - right (55), left (56). For regions with less than 3 samples analysed the frequency was not calculated; symmetrical regions were counted as one for the analysis.

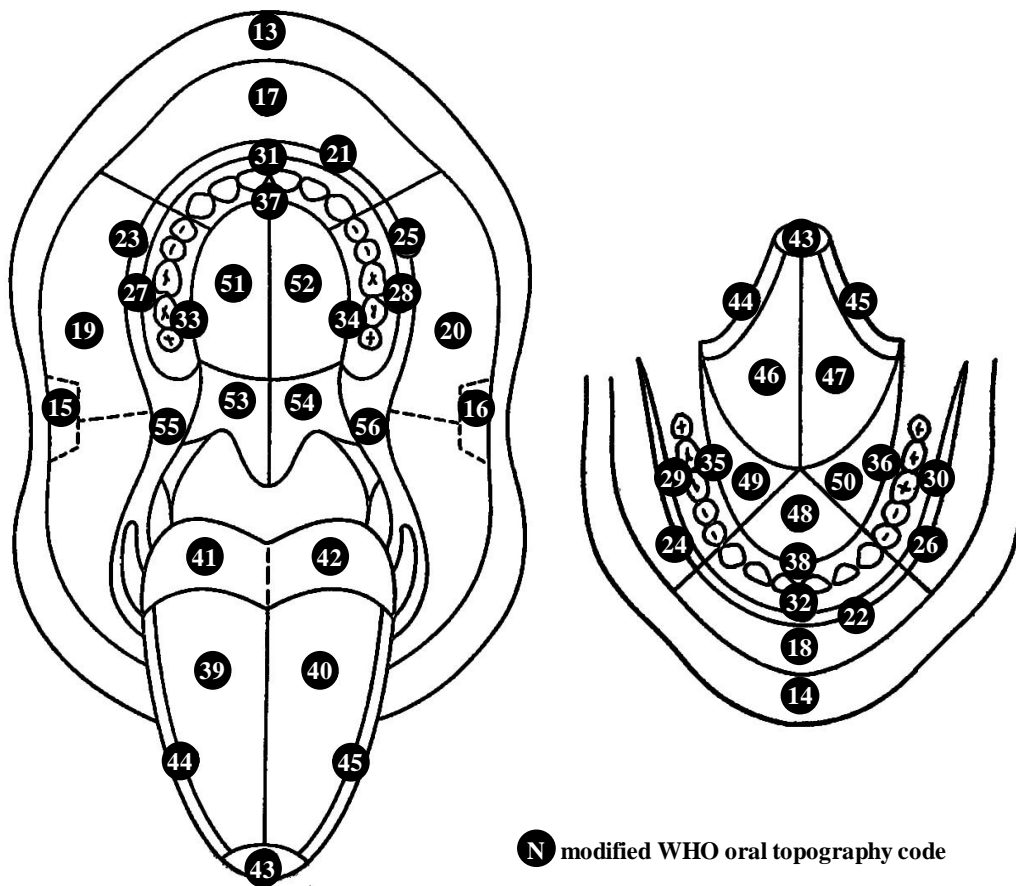


Figure 1. Modified WHO oral topography codes by Roed-Petersen and Roenstrup
 (Kramer et al 1980; Mattila et al 2012) (36)

3.4. HPV detection and genotyping

Samples were collected in TES buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA, pH 7.9; 0.5% SDS), frozen at -20°C, and transported to the laboratory for HPV detection and genotyping. DNA was isolated by the high salt method which allows the precipitation of proteins. Briefly, oral cell suspensions were treated with proteinase K (100 µg/ml) in TES buffer overnight at 37°C or 2 h at 56°C. Then, 1/3 volume of 5 M NaCl was added to the suspension, after 15-30 min incubation at 4°C, and 15 min centrifugation at 4°C, and 14,000g, the supernatant was carefully collected, and treated with 2 volumes of 96% cold (-20°C) ethanol. The precipitation of DNA was facilitated by incubation for at least 2 hours at -20°C, and 15 min at -70°C. The DNA was then collected after 15 min centrifugation at 4°C, and 14,000 g, one washing of excess salt, and a second short centrifugation, and resuspended in ultra pure sterile water (50-100µl). The quality and quantity of DNA was determined spectrophotometrically (73).

Alpha-PV detection by in-house polymerase chain reactions (PCR)

Detection and genotyping of alpha genus HPV types was done as described previously (36).

In house polymerase chain reaction (PCR) were performed using MY09/11 or PGMY09/11 consensus PCR primers followed by type-specific PCR amplifying HPV types 6/11, 16, 18, 31, 33, 45, 52, and 58 in 4 separate multiplex reactions for all samples irrespective of the initial consensus PCR results. Negative or very weakly positive samples by MY/PGMY09/11 and type-specific PCR were further analysed by L1C1/L1C2-1/L1C2-2, and/or GP5+/6+ consensus PCR. Human beta-globin was amplified together with the MY09/11 or PGMY09/11 consensus primers to verify the suitability of the isolated DNA for PCR. The PCR was performed with 5 ng/µl of DNA in each reaction and each run included positive and negative controls (all reaction components except DNA). All standard procedures to avoid DNA contamination were applied.

Beta-PV detection by Luminex

The frequency of different beta/HPV types in cytobrush oral samples were detected by PCR amplification with FAP59/64 consensus primers (74). Furthermore, a subset of samples were additionally analysed for the presence of beta-PV types using type specific, multiplex genotyping assay (TS-MPG assay, IARC, Lyon, France), which combines multiplex PCR with a bead-based Luminex technology (75, 76). This assay detects 43 different beta-PV types, with the human beta-globin gene serving as a control. Each PCR amplification run included positive and negative controls and was set-up in dedicated clean box after UV light decontamination.

Following PCR, 10µl of each reaction mixture was analysed using the Luminex instrument (Luminex Corporation, Austin, TX) as described previously (77, 78) with additional assay negative control well. All runs were assessed for any repeat patterns of HPV positivity on individual run plates.

3.5. Statistical data analysis

Patient data was recorded in the MS Access database and analysed in MS Excel (Microsoft, USA). Chi square test and Chi²test for trend were used to determine statistically significant differences between sample groups and were calculated using Graph Pad Prism (GraphPad software, USA). P values < 0.05 were considered statistically significant.



4. RESULTS

4.1. Description of the study population

In total, there were 343 subjects with oral diagnosis, and 83 control subjects with apparently clinically healthy oral mucosa (Table 1). Oral lesions were initially diagnosed according to clinical features and further confirmed by histopathology analysis. There were 275 women and 151 men. The mean age of the study population was 46.46 years, ranging from 4 to 83 years. The mean age of control samples was 32.16 (15-70 years).

Table 1. Description of the study population

Period of sample collection	From January 13, 2000 to July 9, 2015
Number of cases	426
Oral mucosa changes	343
Control*	83
Gender	
Women	275
Men	151
Age (mean age)	from 4 to 83 (46.46) years
Women	from 4 to 83 (47.69) years
Men	from 4 to 82 (44.21) years

*healthy oral mucosa

Table 2 shows the distribution of individual clinical diagnosis grouped into six categories of diagnosis: 1) premalignant disorders (N=146); 2) benign proliferative lesions (N=99); 3) inflammatory lesions (N=42); 4) ulcerations (N=29); 5) salivary gland diseases (N=21) and 6) carcinoma (N=6). In majority of patients premalignant and benign oral lesions were present.

Table 2. Frequency of individual clinical diagnosis distributed into the main categories

Category of clinical diagnosis	Patients	%
Premalignant	146	34.27
Leukoplakia	73	17.13
Lichen planus	54	12.67
Lichen ruber erosivus	8	1.88
Lichenoid reaction	6	1.41
Erythroplakia	4	0.94
Chelitis actinica	1	0.23
Benign	99	23.24
Papilloma	34	7.98
Verruca vulgaris	28	6.45
Keratosis mucosae	21	4.93
Fibroma	12	2.81
Hyperplasia mucosae	2	0.47
Condyloma acuminata	1	0.23
Solitary pigmentation	1	0.23
Inflammatory	42	9.86
Stomatitis	16	3.75
Glossitis	14	3.28
Chelitis	6	1.41
Stomatitis nicotinic	3	0.70
Candidosis	2	0.47
Gingivitis	1	0.23
Ulcerations	29	6.81
Aphthae	11	2.58
Decubital ulcerations	4	0.94
Stomatitis ulcerosa	3	0.70
Palatitis herpetica	2	0.47
HSV gingivostomatitis	2	0.47
Palatitis erosiva	2	0.47
Herpes labialis	2	0.47
Pemphigoid	1	0.23
Gingivitis ulcerosa	1	0.23
Herpangina	1	0.23
Salivary gland diseases	21	4.93
Sialoadenitis	14	3.28
Mucocele	7	1.65
Carcinoma	6	1.41
OSCC	6	1.41
Control	83	19.48
Grand Total	426	100.00

Table 3 presents the distribution of oral diagnosis category according to gender. Women were twice more frequent than men and have twice more lesions in comparison to men. However, this finding cannot be interpreted as the women are twice as much sicker than men. Finding of increased incidence of oral cancer in men, compared to women, is consistent with epidemiological data (14).

Table 3. Distribution of oral diagnosis by gender sorted descending by frequency

Category of clinical diagnoses*	Women		Men		Total	
	N	%	N	%	N	%
		232	67.64	111	32.36	343
Premalignant	98	67.12	48	32.88	146	34.27
Benign	68	68.69	31	31.31	99	23.24
Inflammatory	31	73.81	11	26.19	42	9.86
Ulcerations	18	62.07	11	37.93	29	6.81
Salivary gland disease	16	76.19	5	23.81	21	4.93
Carcinoma	1	16.67	5	83.33	6	1.41
Control	43	51.81	40	48.19	83	19.48
Total	275	64.55	151	35.45	426	100

* Whenever patient had two or more diagnoses only the most severe diagnosis was taken into account for statistical analysis.

Figure 2 shows the distribution of oral diseases by age groups in all patients. The frequency of premalignant lesions increases with age. The occurrence of oral cancer is also more common in older age, while the occurrence of benign lesions decreases with age although their frequency is almost equal in all age groups.

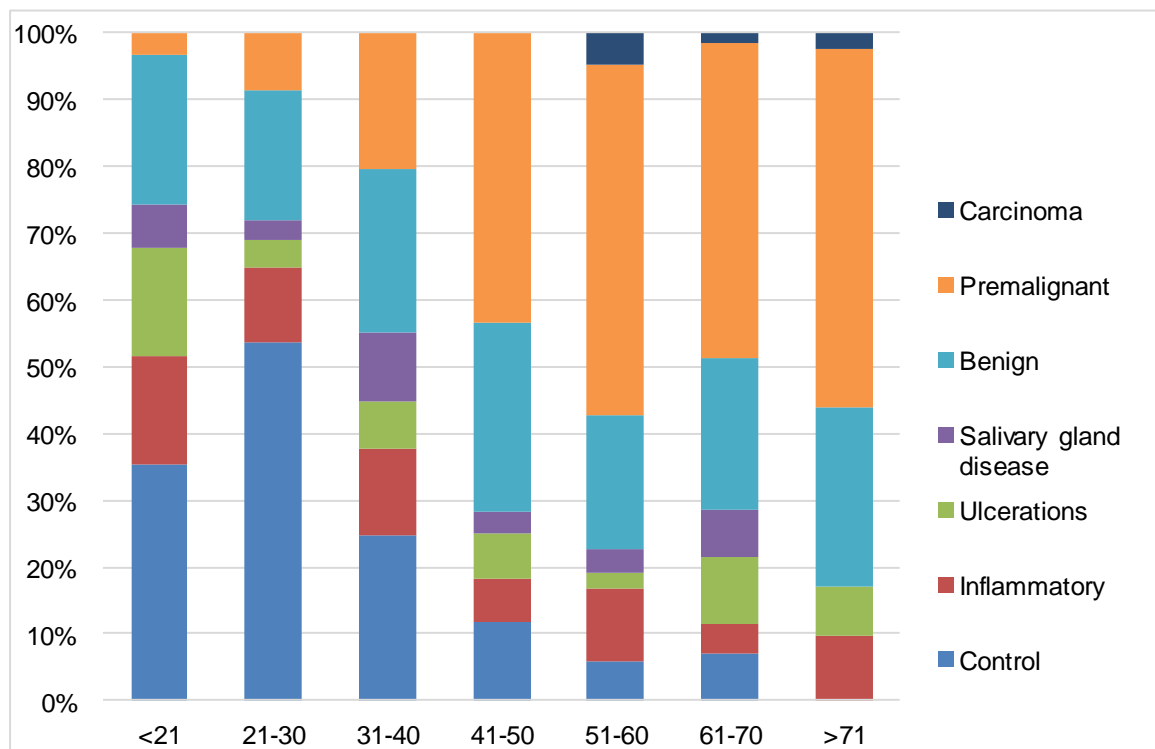


Figure 2. Distribution of oral diagnosis according to age groups

(control = healthy oral mucosa)

4.2. Oral sampling

In 89.92% of subjects, including controls, samples from oral mucosa tested for the presence of HPV types were taken once, and in 10.08% subjects more than twice. In women samples were taken almost as much as in men, only in one man the sample was taken four times and in two women six times (Table 4). Multiple sampling was done with the purpose of monitoring dynamics of HPV infection.

Table 4. Number of oral mucosa sampling per patient and according to gender.

Sample taken	F		M		Total examinees		Total samples
	N	%	N	%	N	%	N
Once	216	89.63	123	90.44	339	89.92	339
Twice	22	9.13	12	8.82	34	9.02	68
Three times	1	0.41	0	0	1	0.27	3
Four times	0	0	1	0.74	1	0.27	4
Six times	2	0.83	0	0	2	0.53	12
Grand Total	241	100	136	100	377	100	426

Table 5. The average number of sampling by groups of diagnoses for all subjects (patients and controls) in which sample was taken once.

Clinical oral diagnosis	No of cases	Average No of sampling
Benign	99	1.12
Carcinoma	6	1.33
Control	83	1.04
Inflammatory	42	1.02
Premalignant	146	1.84
Salivary gland disease	21	1.14
Ulcerations	29	1.07
Grand Total	426	1.34

The analysis of a sampling in both, patients and controls, shows that sampling was taken in all subjects on average 1.34 times, but most frequently in patients with premalignant lesions. In these patients, swabs were taken on average twice (Table 5).

Table 6. Average number of sampling in all subjects according to the category of diagnosis

in which sample was taken more than two times (3x, 4x, and 6x).

Clinical oral diagnosis	No of cases	Average No of sampling
Benign	12	2
Carcinoma	2	2
Control	3	2
Inflammatory	1	2
Premalignant	64	2.92
Salivary gland disease	3	2
Ulcerations	2	2
Grand Total	87	2.68

The samples were taken in all subjects in average 2 times, while in 64 patients with premalignant lesions samples were taken almost 3 times (Table 6), what speaks in favor of regular control of these risk lesions (Table 6).

4.3. HPV status in the core study population

Prevalence of HPV infection in oral lesions and healthy oral mucosa is presented in Table 7. A total of 426 samples from 343 patients and 83 controls collected during the period from 2000 to 2015 were successfully analysed for the presence of HPV DNA. Positive finding HPV DNA was detected in 41.98% samples of oral lesions and in 18.07% control samples of healthy mucosa.

There was a statistical difference in the presence of alpha HPV types* between oral premalignant changes and controls ($p=0.0413$) and between oral benign changes and controls ($p=0.0008$). There is also a statistical difference in the presence of beta HPV types** between oral premalignant changes and controls ($p=0.0093$) and between inflammatory lesions and controls ($p=0.01$).

In majority of lesions and in control samples beta-HPV undetermined type X was more prevalent than LR-HPV. In certain cases positive finding of HR HPV (alpha -HPV) was found predominantly in premalignant and benign proliferative lesions as well as in few cases of patients with ulcerations, salivary gland disease and inflammatory changes of oral mucosa and even in several controls (Figure 3).

Table 7. HPV status in different oral diseases groups

HPV	Positive						Negative		Total	
	Alpha HPV		Beta HPV		Total Positive		N	%	N	%
	N	%	N	%	N	%				
Oral diseases	59	17.2	85	24.78	144	41.98	199	58.02	343	80.52
Premalignant	22	15.07*	39	26.71**	61	41.78	85	58.22	146	34.27
Benign	24	24.24*	20	20.2	44	44.44	55	55.56	99	23.24
Inflammatory	3	7.14	13	30.95**	16	38.1	26	61.9	42	9.86
Ulcerations	4	13.79	5	17.24	9	31.03	20	68.97	29	6.81
Salivary gland disease	5	23.81	6	28.57	11	52.38	10	47.62	21	4.93
Carcinoma	1	16.67	2	33.33	3	50	3	50	6	1.41
Control	5	6.02*	10	12.05**	15	18.07	68	81.93	83	19.48
Grand Total	64	15.02	95	22.3	159	37.32	267	62.68	426	100

* statistical difference in the presence of alpha HPV types

** statistical difference in the presence of beta HPV types

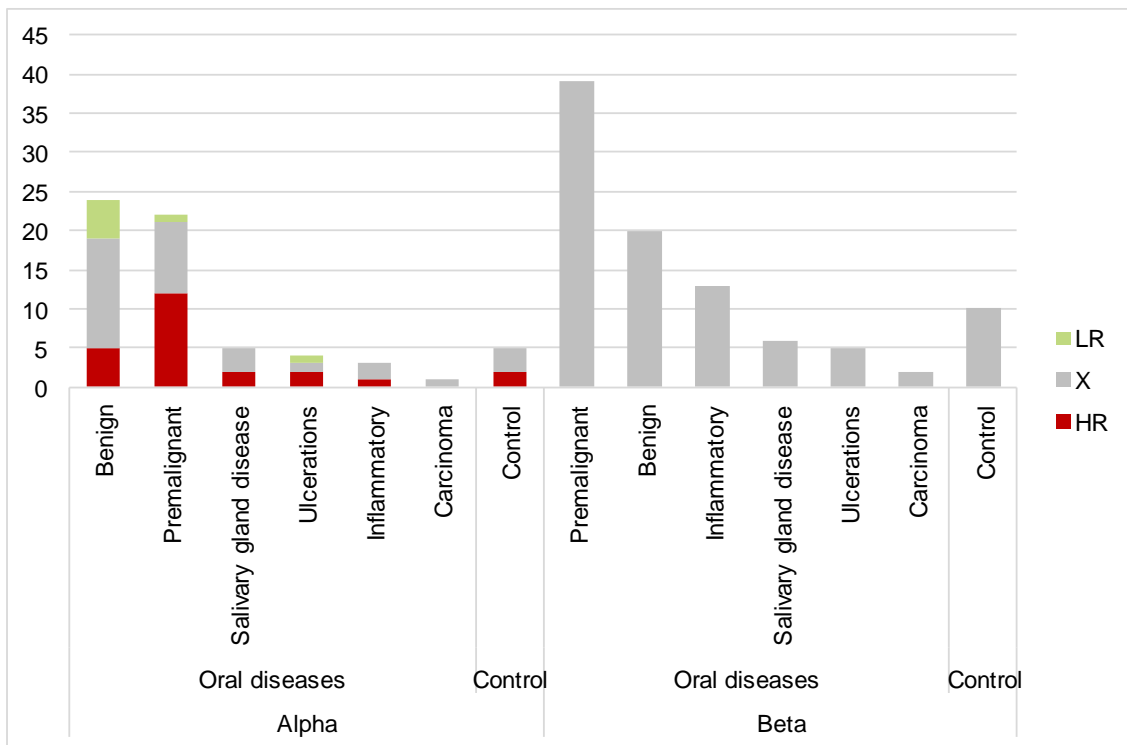


Figure 3. Distribution of HPV risk groups according to oral diseases in HPV positive samples. HR - high risk (HPV 16, 18, 31, 33, 45, 52 and/or 58), LR - low risk (6 or 11) and X - HPV type(s) of undetermined risk, i.e. untyped HPV.

The distribution of HPV types by age is shown in Figure 4. The frequency of beta HPV types prevail among all samples, increases with age and has a peak in middle and older age. However, in all age groups HR HPV (alpha -HPV) are present and are the most common between the ages of 20 and 30 years of age and over the age of 60. The reason for such distribution in younger patients can be sexual behaviour, and in the elderly decreased immunity and medications. Increased frequency of HR HPV types in the sixth and seventh decade of life correlates with greater appearance of premalignant oral lesions and cancer in these age groups as shown on Figure 4.

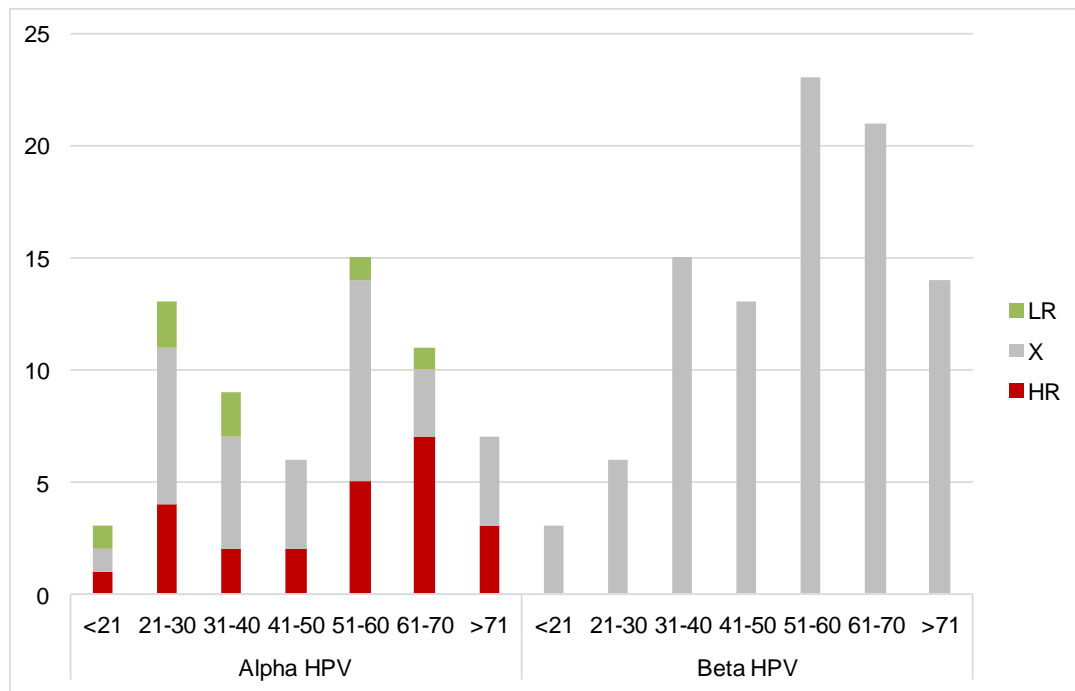


Figure 4. Distribution of HPV risk groups according to age groups; HR – high risk (HPV 16, 18, 31, 33, 45, 52 and/or 58); LR - low risk (HPV 6 or 11); X - HPV type(s) of undetermined risk, i.e. untyped HPV.

4.4. Subset of followed-up patients

Through 15 years of monitoring of all subjects who participated in the study (from 2000-2015), in 38 patients oral mucosal samples assessed for HPV were taken more than twice. The reason for multiple sampling in addition to regular follow up of oral lesions, was also monitoring of dynamics of HPV infection in the oral mucosa with regard to the persistence of infection, change the type of the virus, the progression of lesions associated with virus type and clearance of HPV infection.

Results has showed that out of the 26 initially HPV negative lesions, 5 became HPV positive: of these, infection with HR HPV 52 was proven in one case and in four cases with HPV X. Among five initially positive lesions, in one case the LR HPV 6/11 has been detected and in four cases HPV X, which cleared with time. Two cases that were initially negative, became positive during follow-up: one case with HPV X remained HPV X, while in another case of multiple HPV infection with HPV 6/11, 16 and 18, co-infection disappeared, and a novel infection with HPV 31 occurred. In this person during the follow-up period initial clinical premalignant lesion transformed to malignant change (Table 8).

Table 8. HPV status in followed-up patients with oral mucosa diagnosis

	Final				Total	
	Negative		Positive			
Initial	N	%	N	%	N	%
Negative	26	68.42	5*	13.16	31	81.58
Positive	5**	13.16	2***	5.26	7	18.42
Total	31	81.58	7	18.42	38	100

*one case with HR HPV 52 and 4 cases with HPV X, which occurred with time

**one case with LR HPV 6/11 and 4 with HPV X, which cleared with time

***in one case with HPV X remained HPV X, while in another case of multiple HPV infection with HPV 6/11, 16 and 18 resolved and a novel infection with HPV 31 occurred

By analysing the course of HPV infection in patients with various oral lesions, greatest dynamics of HPV in benign and premalignant lesions was observed. Changes were seen in the a) shift of HPV types (after clearance of primarily infection with HPV 6/11, 16 and 18, new infection with type HPV 31 occurred); b) clearance of infection (with HPV X and HPV 6/11) in minority of cases; c) persistence of infection with the same type (HPV X and HR HPV52) and development of new infection with a new type (HPV X and HPV 31) as shown in Table 9.

Table 9. Dynamic of HPV infection in followed-up patients with oral diseases

HPV infection	Transient	Transient followed by new one	New	Persistent	Continuously negative	Total
Inflammatory	0	0	0	0	1	1
Salivary gland disease	1*	0	0	0	0	1
Ulcerations	0	0	0	0	1	1
Benign	1 *	0	1*	1 [#]	2	5
Pre-malignant	3 ***	1**	2*	1*	20	27
Carcinoma	0	0	1*	0	0	1
Control	0	0	0	0	2	2
Grand Total	5	1	4	2	26	38

* HPV X (different HPV types, other than 6/11, 16, 18, 31, 33, 45, 56 and 58)

** same case primarily HPV 6/11, 16 and 18, then new infection with HPV 31

***one case with HPV X and two cases with HPV 6/11

one case with HR HPV 52

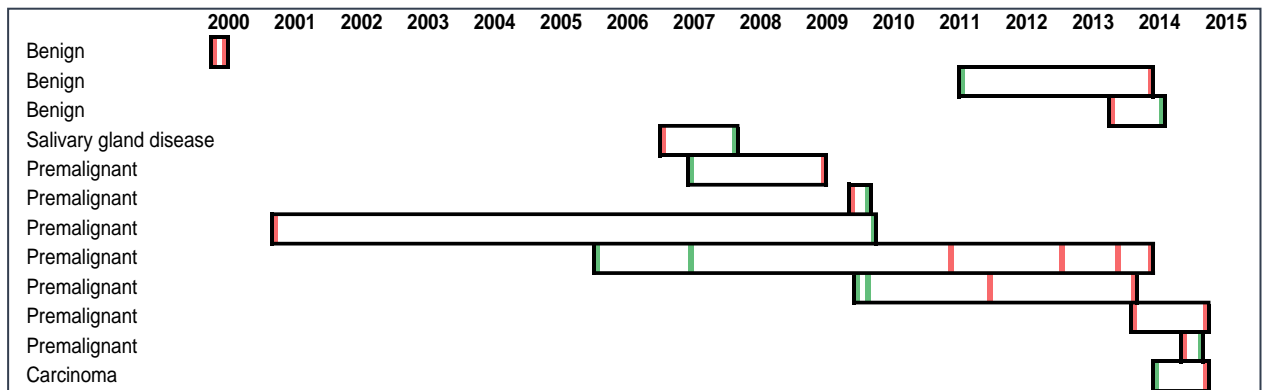


Figure 5. Follow-up of patients with oral mucosa changes and HPV status through years

(green = negative HPV; red = positive HPV) with average infection time being 5.8 months (n=12; 2 cases permanently positive; five initially negative and became positive (-+), and five initially positive became negative (+-); outlier patient with 109 months between tests excluded).

Figure 5 shows the timeline and dynamic course of HPV infection in twelve patients in which the sample was taken more than two times during follow-up period. Four cases with premalignant changes acquired HPV infection, while in the three initially positive infection cleared. In one case of oral carcinoma, lesion was at first visit (before intervention) negative, and became positive on control sampling after intervention during follow up.

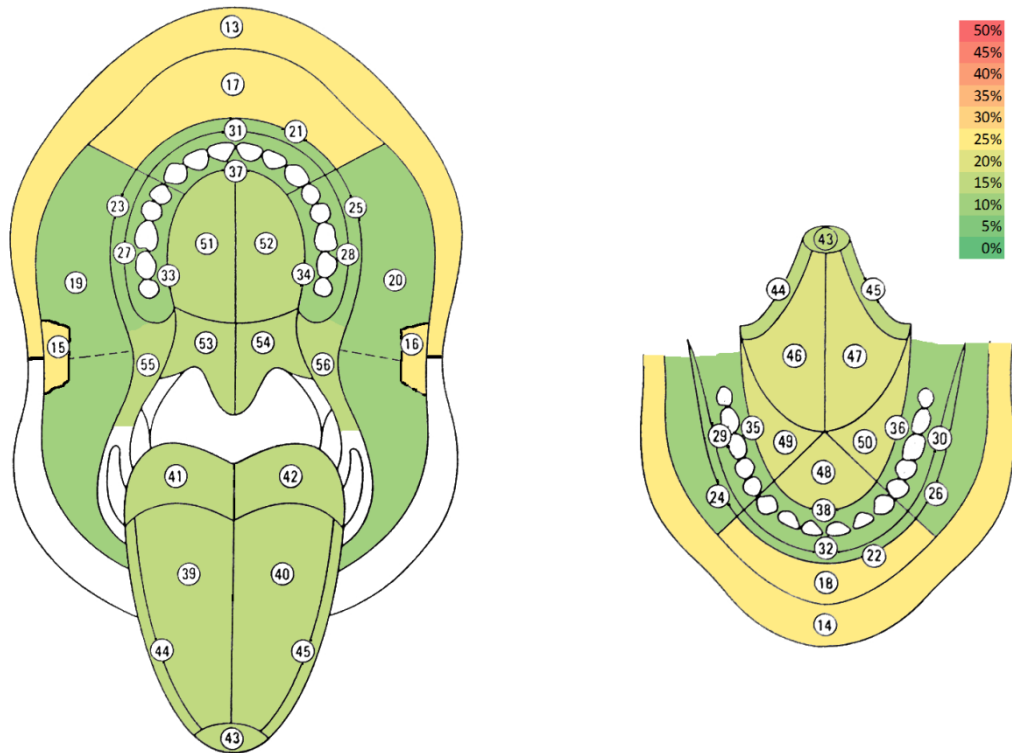
4.5. HPV status according to oral topographical sites

Table 9 and Figure 6 represent topographic distribution of HPV with particular attention to the distribution of alpha and beta genus HPV on oral mucosa irrespective of type of lesion. Each analysed sample had accompanying sampling location information according to the modified WHO oral topography coding (Table 10).

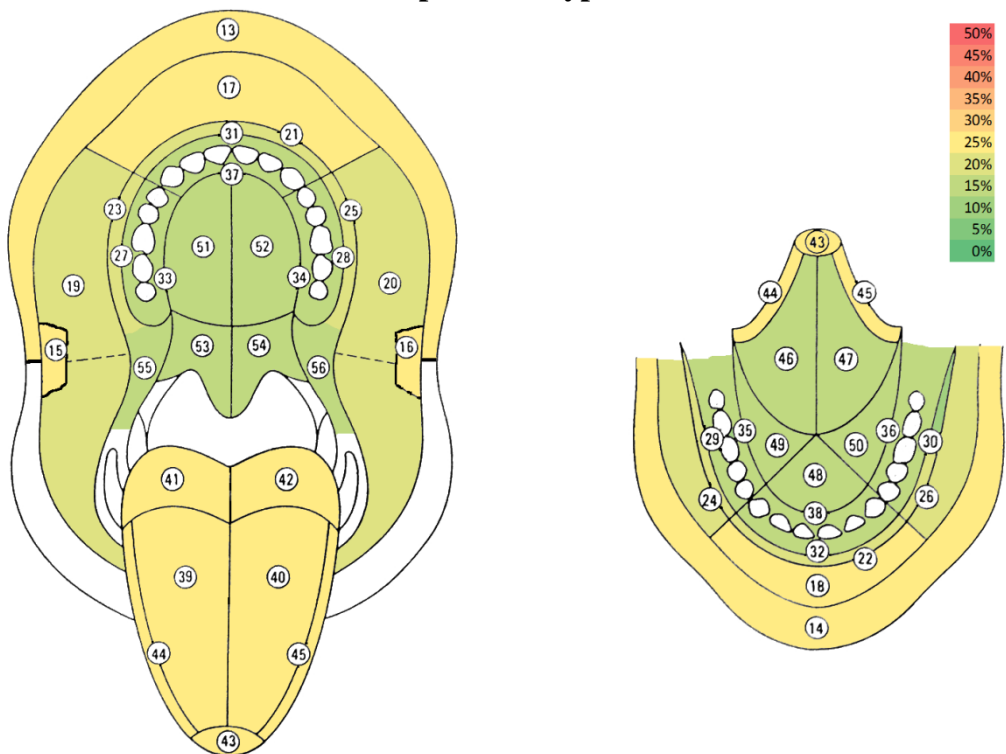
Table 10. Distribution of HPV according to WHO oral topography codes

(by Roed-Petersen and Roenstrup (36).

Region code (WHO)	Location	Total No. cases	Alpha		Beta	
			No	%	No	%
13-18	Labial mucosa	49	12	24.5%	12	24.5%
19-26	Buccal and vestibular mucosa	124	13	10.5%	27	21.8%
27-38	Gingiva	48	5	10.4%	8	16.7%
39-45	Tongue	72	13	18.1%	18	25.0%
46-50	Ventral tongue mucosa and sublingual mucosa	46	10	21.7%	7	15.2%
51-56	Palate	67	12	17.9%	11	16.4%
whole	Whole mucosa	35	7	20.0%	12	34.3%
(blank)	#N/A	154	20	13.0%	26	16.9%



Alpha HPV types



Beta HPV types

Figure 6. Distribution of HPV alpha and beta types in the oral cavity according to WHO oral topography codes (36)

Figure 6 graphically represents the distribution and frequency of alpha and beta genus HPV positivity at different topographical sites in the oral cavity. The affinity of a particular genus type HPV for certain topographical areas of the oral mucosa is observed.

Alpha HPV types were found in the highest percentage in the anterior parts of the oral cavity: on the labial mucosa, commissure of the lips and vestibular oral mucosa, ventral tongue and sublingual mucosa. Such distribution of alpha HPV types show affinity for risk localizations in the mouth (commissure of the lips and ventral tongue and sublingual mucosa) and may have prognostic significance.

Beta HPV types were in general more frequently detected compared to alpha types. They were found in wider areas of the oral cavity especially on the labial, buccal and vestibular mucosa including commissure of the lips, mucosa of the dorsum of the tongue and the lateral edges of the tongue where alpha HPV types are detected to a much lesser extent.



5. DISCUSSION

The association between HR HPV types and malignant lesions has been shown in many studies (4, 28, 29). HR HPV types are known to be potential etiologic factor in oral carcinoma and considered to be initiator epithelial proliferation (4). Although the association between anogenital HPV infections and anal and genital cancer is indisputable (12) yet controversy surrounds regarding their role in oropharyngeal malignant and premalignant lesions (7-10).

This study is the only study on both alpha- and beta-HPV type prevalence in oral mucosal lesions in the Croatian population and also the only one that was following HPV positive and negative lesions for a longer period of time in order to get better understanding of the relationship between HPV infection and different oral lesions. Although the collection of the study population took 16 years, the advantage of this study is its sample size and uniformity of material, sampling, and further processing procedures. All the oral scrapings were taken in the single clinic under the same conditions, and DNA preparation and HPV analysis was also done in the same manner in the research virological laboratory. Clinical diagnosis of the oral lesions and sampling was evaluated by a single specialist. In each case, oral epithelial cells were taken with the cytobrush and biopsies were excluded from this study. In addition, each specimen was classified according to the WHO topography code (79) in order to evaluate the relationship of HPV infection and oral lesions at the different locations of the mouth.

Three sets of consensus primers were used to maximize the identification of alpha-HPV DNA. In addition, genotyping for specific alpha-HPV types was performed on all samples regardless of consensus PCR result to further increase the HPV detection sensitivity. Types selected for genotyping were chosen due to their abundance in cervical carcinoma in the world (65). Similar studies of HPV detection in oral cells by PCR were done earlier confirming the significant association of HPV positivity in oral carcinoma, potentially malignant disorders such as oral leukoplakia, oral lichen ruber planus, and epithelial dysplasia

(38). This study includes all the listed oral lesions and only one lesion progressed in oral carcinoma.

The HPV prevalence in healthy oral mucosa (n=15) and in oral lesions (n=144) is higher in men than in women both in our study and the literature (80, 81). However, regarding the different morphology and diagnosis, herein no statistically significant difference was found because of the low number of study subgroups.

As expected, we found statistical difference in the presence of alpha-HPV types between oral premalignant changes 15.07% and controls 6.02% ($p=0.0413$) and between oral benign changes 24.24% and controls ($p=0.0008$). Furthermore, there was also statistical difference in the presence of beta-HPV types between oral premalignant changes 24.78% and controls 12.05% ($p=0.0093$) and between inflammatory lesions 30.95% and controls ($p=0.01$). These results suggest a significant association between the presence of premalignant oral lesions and benign (papilloma) and positive results for presence of oral HPV. Most of the studies that were investigating prevalence of HPV in oral lesions were focused on investigation of HPV prevalence in oral lesions that are known to be associated with HPV infection (oral papilloma, and precancerous and cancerous lesions) (27, 28, 33, 46). In our study we included inflammatory disease and salivary gland diseases and we got significantly higher prevalence between these two groups compared with control group. One of the possible explanations for the observed data is that saliva play important role in behaviour of oral HPV infection. Reduced salivary flow makes oral mucosa more susceptible to micro trauma and infection (82) that is prerequisite for HPV infection.

The prevalence of HPV infection in oral lesions in our study agrees with the findings of Miller and Johnstone (28) who found HPV infection rate two to three times higher in patients with precancerous oral lesions compared with healthy mucosa. Our results reinforce the hypothesis of possible causal role of HPV infection, especially HR HPV infection, in pathogenesis of some cases of oral premalignant lesions. The most common HPV type in premalignant oral lesion was HR HPV type that is similar to the results of other studies (22, 28, 32). Control samples of clinically healthy oral mucosa were HPV positive in 18.07% that is higher than results that Kreimer et al. (83). In their systemic review, they found 4.5% overall HPV positivity in healthy subjects, of which 1.3% had HPV 16.

Based on our result HPV infection can affect any age group even though it seems to be most common between the age 20 and 30 years and over the age of 60. The reason for such distribution in younger patients can be sexual behaviour, and in the elderly decreased immunity and medications. Increased frequency of HR HPV types in the sixth and seventh decade of life correlates with greater appearance of premalignant oral lesions and cancer in these age group. Most of the infection were transient in our study that is similar to the findings on cervical epithelium (45); 60-70% of cervical infection clearing within 1 year and 91% clearing within 2 years. HR HPV types have been shown to persist longer than LR HPV types (45).

Our results showed that topography plays a role in HPV prevalence in oral lesions. Even though the same lesions were found in different regions of the oral cavity, the HPV positivity was higher in specific topographical regions irrespective of diagnosis. It was not possible to analyse different diagnosis-topography combinations due to small sample subgroups, thus the data were grouped according to HPV frequency associated with the diagnosis and topography of lesions. The statistical analysis has shown that HPV positivity of the lesions that belong to the moderate or high HPV frequency-diagnosis subgroups is significantly associated with location. The topography was not significantly associated with low HPV frequency-diagnosis subgroup as the number of HPV positive samples was very small, but it was significantly associated with the moderate HPV frequency topography subgroup. Undoubtedly, it is true

that some particular diagnoses are more associated with HPV and thus more often HPV positive, however, these data show that topography of the lesion is significantly associated to the HPV positivity of a particular lesion, and even somewhat more than the diagnosis. One of the possible explanations for the observed data is that the locations with the highest HPV positivity (vermillion border, labial mucosa and commissures, sublingual mucosa, ventrum of the tongue and lateral margins) are the most frequently exposed sites to micro trauma that is prerequisite for HPV infection transmission and these sites also pose the highest risk for malignant transformation.



6. CONCLUSION

Based on the obtained result of the study we can conclude the following:

1. the prevalence of different HPV types in different oral lesions in the cohort of oral medicine patients is significantly higher than in controls with healthy oral mucosa,
2. oral HPV infection shows dynamic course and is transient in nature,
3. premalignant HR HPV-associated lesion progressed to a more severe form of disease in one patient,
4. during long term follow-up of HPV infection on oral mucosa, the types of HPV are often changing from one type to another, as shown in cases in which the primary infection was resolved and new one was acquired,
5. the distribution of alpha-HPV and beta-HPV types on oral mucosa may have prognostic significance, since both HPV genus types affect mainly the mucosa of anterior parts of the mouth and HR HPV types of alpha genus has affinity for particular oral localizations which pose a risk for malignant transformation,
6. the natural history of the oral HPV infection and its association with oral premalignant and proliferative lesions as shown in this study, indicates the need for regular oral assessment and HPV control in patients with initially positive findings on oral mucosa.



7. LITERATURE

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1. De Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. *Virology* 2004; 324:17-27.
 2. zur Hausen H, Meinhof W, Scheiber W, Bornkamm GW. Attempts to detect virus-specific DNA in human tumors. I. Nucleic acid hybridizations with complementary RNA of human wart virus. *Int J Cancer* 1974; 13:650-6.
 3. zur Hausen H. Condylomata acuminata and human genital cancer. *Cancer Res* 1976; 36 (2 pt 2):794.
 4. zur Hausen H. Human papillomaviruses and their possible role in squamous cell carcinomas. *Curr Top Microbiol Immunol* 1977; 78:1-30.
 5. Walboomers J M, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol*, 1999;189:12-19
 6. Abramowitz L, Jacquard AC, Jaroud F, et al. Human papillomavirus genotype distribution in anal cancer in France: the EDiTHV study. *Int J Cancer* 2011; 129:433-439.
 7. De Vuyst H, Clifford GM, Nascimento MC, et al. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. *Int J Cancer* 2009;124:1626-1636.
 8. Miralles-Guri C, Bruni L, Cubilla AL, et al. Human papillomavirus prevalence and type distribution in penile carcinoma. *J Clin Pathol* 2009; 62:870-878.
 9. Guily S, Lacau J, Jacquard AC, et al. Human papillomavirus genotype distribution in oropharynx and oral cavity cancer in France-the EDiTH VI study. *J Clin Virol* 2011;51:100-104.
 10. Doorbar J, Quint W, Banks L, et al. The Biology and Life-Cycle of Human Papillomaviruses *Vaccine* 2012;30:F55-F70.
 11. Syrjänen S, Rautava J. HPV and Cancer. *Duodecim*. 2015; 131 (19): 1765-74.
 12. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Human papillomaviruses, International Agency for Research on Cancer, World Health Organization, Lyon, France 2007:1-690.
 13. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Human papillomaviruses, International Agency for Research on Cancer, World Health Organization, Lyon, France 1995:1-436.

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14. Franceschi S, Munoz N, Bosch XF, et al. Human papillomavirus and cancers of the upper aerodigestive tract: a review of epidemiological and experimental evidence. *Cancer Epidemiol Biomarkers Prev* 1996;5:567-575.
 15. Bouvard V, Baan R, Straif K, et al. A review of Human carcinogens-part B: biological agents *Lancet Oncol* 2009; 10:321-322.
 16. Grce M, Sabol I, Milutin Gašperov N. Burden and prevention of HPV related disease: Situation in Croatia. *Period Biol* 2012; 114 (4): 479-487.
 17. Grce M, Mravak-Stipetić M. Human papillomavirus-associated disease. *Clin Dermatol*. 2014; 32(2):253-8.
 18. Praetorius-Clausen F. Geographical aspects of oral focal epithelial hyperplasia. *Pathol Microbiol (Basel)* 1973; 39:204-213.
 19. Said AK, Leao JC, Fedele S, et al. Focal epithelial hyperplasia-an update. *J Oral Pathol Med* 2013; 42:435-442.
 20. Praetorius-Clausen F, Mogeltoft M, Roed-Petersen B, et al. Focal epithelial hyperplasia of the oral mucosa in a south-west Greenlandic population. *Scand J Dent Res* 1970;78:287-294.
 21. Bonagura VR, Hatam LJ, Rosenthal DW, et al. Recurrent respiratory papillomatosis: a complex defect in immune responsiveness to human papillomavirus-6 and 11. *APMIS* 2010;118:455-470.
 22. Gupta S, Gupta S. Role of human papillomavirus in oral squamous cell carcinoma and oral potentially malignant disorders: A review of the literature. *Indian Journal of Dentistry* June 2015;6:91-98.
 23. Mravak-Stipetić M, Lončar-Brzak B, Bakale-Hodak I, Sabol I, Seiwerth S, Majstorović M, Grce M. Clinopathologic correlation of oral lichen planus and oral lichenoid lesions: a preliminary study. *Sci World J*. 2014; 746874. doi: 10.1155/2014/746874
 24. Neville BW, Damm DD, Allen CM, et al. *Oral pathology*. 3rd edition. St Louis: Saunders Elsevier: 2009; p 362.
 25. Grce M. [Molekularna dijagnostika oralnih infekcija]. *Acta Med Croatica*. 2013;67(5):425-32.
 26. Ostwald C, Rutsatz K, Schweder J, et al. Human papillomavirus 6, 11, 16, and 18 in oral carcinomas and benign oral lesions. *Med Microbiol Immunol* 2003; 192:145-8.
 27. Nielsen H, Norrild B, Vedtofte P, et al. Human papillomavirus in human premalignant lesions. *Eur J Cancer B Oral Oncol* 1996;32:264-70.

-
28. Miller CS, Johnstone BM. Human papillomavirus as a risk factor for oral squamous cell carcinoma: a meta-analysis, 1982-1997. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2001; 91:622-36.
 29. Luo CW, Roan CH, Liu CJ. Human papilloma virus in oral squamous cell carcinoma and precancerous lesions detected by PCR-based gene-chip array. *Int J Oral Maxillofac Surg* 2007; 36:153-8.
 30. Feller L, Lemmer J. Oral leukoplakia as it relates to HPV infection: a review. *Int J Dent* 2012:540561.
 31. Evans MF, Matthews A, Kandil D, et al. Discrimination of 'driver' and 'passenger' HPV in tonsillar carcinomas by the polymerase chain reaction, chromogenic in situ hybridization, and p16INK4a immunohistochemistry. *Head Neck Pathol* 2011;5:344-8
 32. Szarka K, Tar I, Feher E, et al. Progressive increase of human papillomavirus carriage rates in potentially malignant and malignant oral disorders with increasing malignant potential. *Oral Microbiol Immunol* 2009; 24:314-318.
 33. Gassenmaier A, Hornstein OP. Presence of human papillomavirus DNA in benign and precancerous oral leukoplakias and squamous cell carcinomas. *Dermatologica* 1988; 176:224-233
 34. Reichart PA, Philipsen HP. Oral erythroplakia-a review. *Oral Oncol* 2005; 41:551-561.
 35. Nielsen H, Norrild B, Vedtofte P, et al. Human papillomavirus in oral premalignant lesions. *Eur J Cancer B Oral Oncol* 1996; 32:264-270.
 36. Mravak-Stipetić M, Sabol I, Kranjčić J, Knežević M, Grce M. Human Papillomavirus in the Lesions of the Oral Mucosa According to Topography. *PLoS ONE* 2013; 8(7); e69736. doi:10.1371/journal.pone.0069736.
 37. Sellors J, Sankaranarayanan R. Colposcopy and treatment on cervical intraepithelial neoplasia: a beginners manual. Lyon; 2003.
 38. Syrjanen S, Lodi G, von Bultzingslowen I, Aliko A, Arduino P, Campisi G, et al. Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review. *Oral Dis* 2011;17(Suppl. 1):58-72.
 39. Gorsky M, Epstein JB. Oral lichen planus: malignant transformation and human papilloma virus: a review of potential clinical implications. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011;111:461-4.

-
40. Syrjanen K, Syrjanen S, Lamberg M, et al. Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. *Int J Oral Surg* 1983;12:418-424.
 41. Arbyn M, de Sanjose S, Saraiya M, et al. EUROGIN 2011 roadmap on prevention and treatment of HPV-related disease. *Int J Cancer* 2012;131:1969-1982.
 42. Leemans CR, Braakhuis BJM, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer* 2010;11:9-22.
 43. Palmieri A, Scapoli L, Martinelli M, Pezzetti F, Girardi A, Spinelli G, et al. Incidence of low risk human papillomavirus in oral cancer: A real time PCR study on 278 patients. *Int J Immunopathol Pharmacol* 2011;24:83-7.
 44. Balaram P, Nalinakumari KR, Abraham E, Balan A, Hareendram NK, Bernard HU, et al. Human papillomaviruses in 91 oral cancers from Indian betel quid chewers-High prevalence and multiplicity of infections. *Int J Cancer* 1995;61:450-4.
 45. Jimenez C, Correnti M, Salma N, Cavazza ME, Perrone M. Detection of human papillomavirus DNA in benign oral squamous epithelial lesions in Venezuela. *J Oral Pathol Med* 2001;30:385-8.
 46. Campisi G, Panzarella V, Giuliani M, Lajolo C, Di Fede O, Falaschini S, et al. Human papillomavirus: Its identity and controversial role in oral oncogenesis, premalignant and malignant lesions (review). *Int J Oncol* 2007;30:813-23.
 47. Feller L, Lemmer J. Oral Leukoplakia as It Relates to HPV Infection: A Review. *International Journal of Dentistry* 2012:540561.
 48. Tanaka T, Tanaka M, Tanaka T. Oral Carcinogenesis and Oral Cancer Chemoprevention: A Review. *Pathology Research International* 2011: 1-10.
 49. Schepman KP, van der Meij EH, Smeele LE, van der Waall. Malignant transformation of oral leukoplakia: a follow-up study of a hospital-based population of 166 patients with oral leukoplakia from The Netherlands. *Oral Oncol* 1998; 34:270-5.
 50. Saghracanian N, Ghazvini K, Babakoochi S et al. Low prevalence of high risk genotypes of human papillomavirus in normal oral mucosa, oral leukoplakia and verrucous carcinoma. *Acta Odontologica Scandinavia*. 2011;69:406-409.
 51. Qingyue W, Chegnming G. Human Papillomavirus in People with Oral Leukoplakia in Guilin area: *Journal of Oral Science Research*; 2006;22(4):443-442.

-
52. Llamas-Martinez S, Esparza-Gomez G, Campo-Trapero J. Genotypic determination by PCR-RFLP of human papillomavirus in normal oral mucosa, oral leukoplakia and oral squamous carcinomas samples in Madrid (Spain). *Anticancer Research* 2008; 28:3733-3742.
 53. Kristoffersen A.K, Enersen M, Kvendokk E et al. Human papillomavirus subtypes in oral lesions compared to healthy oral mucosa. *J Clin Virol.* 2012;53(4)364-6.
 54. Asok M, Mody N, Mehendra R, Patait R et al. Prevalence and relationship of human papillomavirus type 16 and type 18 with oral squamous cell carcinoma and oral leukoplakia in fresh scrapings: a PCR study. *Indian Journal of Medical Science.* 2011;65(5):221-21.
 55. Hou Wei Qiangian S, Xisoming X, et al. Primary study of HPV 16 and HCMV infections in Oral squamous cell carcinoma. *J Oral pathol* 1996;12(2):73-70.
 56. Ai-e H. Human papillomavirus and human cytomegalovirus in oral carcinoma. *J Clin Stomatol.*2008; 24(9):529-528.
 57. Sikka S, Sikka P. Association of Human Papilloma Virus 16 Infection and p53 Polymorphism among Tobacco using Oral Leukoplakia Patients; A Clinicopathologic and Genotypic Study. *Int J Prev Med.* 2014 ;5(4):430-438.
 58. D'Souza G, Agrawal Y, Halpern J, Bodison S, Gillison ML. Oral sexual behaviors associated with prevalent oral human papillomavirus infection. *J Infect Dis* 2009;199:1263-9.
 59. Ragin C, Edwards R, Larkins-Pettigrew M, Taioli E, Eckstein S, Thurman N, et al. Oral HPV infection and sexuality: A cross-sectional study in women. *Int J Mol Sci* 2011; 12:3928-40.
 60. Kreimer AR, Alberg AJ, Daniel R, Gravitt PE, Viscidi R, Garrett ES, et al. Oral human papillomavirus infection in adults is associated with sexual behavior and HIV serostatus. *J Infect Dis* 2004;189:686-98.
 61. Dunne EF, Unger ER, Sternberg M, et al. Prevalence of HPV infection among females in the United States. *JAMA* 2007; 297:813-819.
 62. Muñoz N, Bosch FX, De Sanjosé S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *New Engl J Med* 2003;348: 518-527.
 63. Bosch FX, Lorincz A, Muñoz N, et al. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol* 2002;55:244-265.

-
64. De Sanjose S, Quint W, Alemany L, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol*, 2010; 11:1048-1056.
 65. Kreimer AR, Clifford GM, Boyle P, et al. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prev* 2005; 14: 467-475.
 66. Medeleine MM, Anttila T, Schwartz SM, et al. Risk of cervical cancer associated with Chlamydia trachomatis antibodies by histology, HPV type and HPV cofactors. *Int J Cancer* 2007;120:650-655.
 67. Grce M, Sabol I, Milutin Gašparev N. The transforming properties of human papillomavirus oncoproteins. *Period Biol* 2012; 114 (4): 479-487.
 68. Moody C A, Laimins L A. Human papillomavirus oncoproteins: pathways to transformation. *Nat Rev Cancer* (2010); 10: 550-560.
 69. McLaughlin-Drubin M E, Múnger K. The human papillomavirus E7 oncoprotein. *Virology* (2009); 384:335-344.
 70. Múnger K, Phelps W C, Bubb V, Howley P M, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989; 63: 4417-4421.
 71. Pim D, Banks L. Interaction of viral oncoproteins with cellular target molecules: infection with high-risk vs low-risk human papillomaviruses. *APMIS* 2010; 118:471-493.
 72. Brehm A, NieIsen S J, Miska E A, McCance D J, Reid J L, Bannister A J, Kouzarides T. The E7 oncoprotein associates with Mi2 and histone deacetylase to promote cell growth. *EMBO J* 1999; 18: 2449-2458.
 73. Milutin-Gasperov N, Sabol I, Halec G, Matovina M, Grce M. Retrospective study of the prevalence of high-risk human papillomaviruses among Croatian women. *Coll Antropol* 2007; 31 Suppl 2: 89-96.
 74. Forslund O, A. Antonsson, P. Nordin, B. Stenquist, and B. Goran Hansson. "A Broad Range of Human Papillomavirus Types Detected with a General PCR Method Suitable for Analysis of Cutaneous Tumours and Normal Skin." *J of Gen Virol* 1999; 80(9):24-37.

-
75. Gheit T, Billoud G, de Koning MNC, Gemignani F, Forslund O, Sylla BS, et al. Development of sensitive and specific multiplex PCR method combined with DNA microarray primer extension to detect Betapapillomavirus types. *J. Clin. Microbiol* 2007; 45:2537-2544.
 76. Gheit T, Landi S, Gemignani F, Snijders PJF, Vaccarella S, Franceschi S, et al. Development of a sensitive and specific assay combining multiplex PCR and DNA microarray primer extension to detect high-risk mucosal human papillomavirus types. *J.Clin.Microbiol* 2006 ;44:2025-2031.
 77. Schmitt M, Dondog B, Waterboer T, Pawlita M, Tommasino M, Gheit T. Abundance of multiple high-Risk human papillomavirus (HPV) infections found in cervical cells analyzed by use of an ultrasensitive HPV genotyping assay. *J. Clin. Microbiol* 2010; 48: 143-149.
 78. Schmitt M, Bravo IG, Snijders PJF, Gissmann L, Pawlita M, Waterboer T. Bead-based multiplex genotyping of human papillomaviruses. *J. Clin. Microbiol* 2006; 44:504-512.
 79. Kramer IR, Pindborg JJ, Bezroukov V, Infirri JS. Guide to epidemiology and diagnosis of oral mucosal diseases and conditions. World Health Organization. *Community Dent Oral Epidemiol* 1980; 8: 1-16.
 80. Kojima A, Maeda H, Kurahashi N, Sakagami G, Kubo K, et al. Human papillomaviruses in the normal oral cavity of children in Japan. *Oral Oncol* 2003;39:821-828.
 81. Herrero R, Castellsagué X, Pawlita M, Lissowska J, Kee F, et al. Human Papillomavirus and Oral Cancer: The International Agency for Research on Cancer Multicenter Study. *J Natl Cancer Inst* 2003;95: 1772-1783.
 82. Jensen SB, Vissinik A. Salivary Gland Dysfunction and Xerostomia in Sjögren's Syndrome. *Oral Maxillofacial Surg Clin In Am* 2014;26:35-53.
 83. Kreimer AR, Bhatia RK, Messegue AL, González P, Herrero R, Giuliano AR. Oral human papillomavirus in healthy individuals: a systematic review of the literature, *Sex. Transm. Dis* 2010; 37:386-391.



8. CURRICULUM VITAE

Marjana Knežević was born on January 12th 1976 in Dobož, Bosnia and Herzegovina. She graduated from the School of Dental Medicine University of Zagreb in 2001. In 2002 she started Postgraduate study at the same University. In 2008 she successfully defended her Master degree Thesis entitled “Human papillomaviruses in lesions of the oral mucosa” under the supervision of professor Marinka Mravak-Stipetić DMD, MSc, PhD and Magdalena Grce, PhD. In 2012 she graduated from the School of Dental Medicine University of Pennsylvania where she works as part-time faculty at the Department of Restorative Dentistry and in her private dental office in Philadelphia.

Current Contents Publications:

1. Mravak-Stipetic M, Sabol I, Kranjic J, Knezevic M, Grce M. Human Papillomavirus in the Lesions of the Oral Mucosa According to Topography. PLOS one. 2013; 8(7): e69736.
2. Vodanovic M, Peros K, Zukanovic A, Knezevic M. Periodontal diseases at the transition from the late antique to the early mediaeval period in Croatia. Arch Oral Biol 2012; 57: 1362-1376.
3. Grce M, Knezevic M, Sabol I, Milutin Gasperov N, Mravak-Stipetic M. Association of human papillomaviruse (HPV) and different lesions of oral cavity. Abstract Book of the 24th International Conference and Clinical Workshop. Zheng YM, Qiao X, Song G (ur). Peking, 2007.
4. Lukac J, Mravak-Stipetic M, Knezevic M, Vreck J, Sistig S, Ledinsky M, Kusic Z. Phagocytic functions of salivary neutrophils in the oral mucous membrane diseases. J Oral Pathol Med 2003;32(5):271-4.