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HPV's Impact on Head and Neck Cancers: Exploring Prevalence and Prognostic Significance

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

Sector Group – Medical and Health Sciences
Sector – Clinical Medicine
Sub-Sector – Otorhinolaryngology

Riga, 2024

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The Doctoral Thesis was developed at the Institute of Anatomy and Anthropology, the Institute of Microbiology and Virology of Rīga Stradiņš University, Latvia

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Abbreviations used in the Thesis

ChT	Chemotherapy
DSS	Disease-specific survival
FFPE	formalin-fixed paraffin-embedded
HNSCC	Head and neck squamous cell carcinoma
HPSCC	Hypopharyngeal squamous cell carcinoma
HPV	Human papillomavirus
HR-	High-risk
IHC	Immunohistochemical, -ly, immunohistochemistry
KSCC	Keratinizing squamous cell carcinoma
LR-	Low-risk
LSCC	Laryngeal squamous cell carcinoma
NKSCC	Nonkeratinizing squamous cell carcinoma
OP	Surgical treatment
OPSCC	Oropharyngeal squamous cell carcinoma
OS	Overall survival
RT	Radiotherapy
UICC	International Union against Cancer

Introduction

Squamous cell carcinoma is the most common malignant tumour in the head and neck region (Sung et al., 2021). Head and neck squamous cell carcinoma (HNSCC) is the seventh most prevalent cancer worldwide, with over 660,000 new cases and 325,000 fatalities occurring each year (Gormley et al., 2022). According to the GLOBOCAN data, 98,412 new cases of oropharyngeal (OPSCC), 98,412 new cases of laryngeal (LSCC), and 84,254 new cases of hypopharyngeal squamous cell carcinomas (HPSCC) were registered in 2020 (Sung et al., 2021).

Tobacco smoking and alcohol consumption are significant synergistic risk factors for the development of HNSCC (Kuper et al., 2002; Hashibe et al., 2009). Cases caused by these factors often exhibit p53 gene mutations involved in cell cycle regulation (Carlos de Vicente et al., 2004). Mutations in the p16 tumour suppressor gene also occur, leading to the loss of p16 function as a CDK inhibitor (Beck et al., 2017; Deneka et al., 2022). Normally, p16 binds to the CDK4/CDK6 complex, suppressing pRb. Disruption of the p16 function results in dysregulation of the cell cycle, leading to uncontrolled cell proliferation (Kotake et al., 2015; Senga & Grose, 2021). Despite an overall decrease in HNSCC incidence over the past 20 years, there has been an increase in the incidence of oral and oropharyngeal squamous cell carcinomas (Taylor et al., 2021; Kawakita et al., 2022).

In addition to these traditional risk factors, high-risk human papillomavirus (HR-HPV) types, especially HPV16, are considered separate and independent risk factors for HNSCC, particularly associated with OPSCC. HPV status has also been associated with the pathogenesis of oral squamous cell carcinoma, but the association between HPV and OPSCC is the strongest (Chaturvedi et al., 2011; Gillison et al., 2015).

While HR-HPV infection, particularly HPV16, is strongly linked to the development of OPSCC with HPV prevalence being as high as 70 % (Schache et al., 2016; Timbang et al., 2019), the role of HR-HPV in other head and neck cancers such as LSCC and HPSCC is still a subject of debate, as these cancers tend to be HPV-negative more frequently and are studied less frequently when compared to OPSCC.

HPV-positive head and neck cancers differ significantly from HPV-negative cancers in terms of molecular mechanisms, tumour progression, epidemiology, and patient survival. The presence of HPV, especially in OPSCCs, serves as a prognostic factor associated with a reduced risk of death and recurrence (Mallen-St Clair et al., 2016). HPV is strongly linked to tonsil cancer, moderately associated with oropharyngeal cancer, and weakly associated with oral cancer (Hobbs et al., 2006). HPV16 seropositivity increases the risk of OPSCC in both smokers and alcohol users, as well as non-smokers and non-alcohol users (D'Souza et al., 2007). However, the definitive association between HPV status and survival outcomes is not established for other types of HNSCCs (H. Wang et al., 2019; Wu et al., 2021; Sharkey Ochoa et al., 2022). HPV-positive HNSCCs exhibit distinct molecular signatures, including wild-type p53 degradation, absence of p53 gene mutations, decreased pRb expression, and subsequent increased p16 expression. These molecular differences can aid in distinguishing HPV-associated cancers, guiding treatment adjustments, and serving as prognostic markers (Mallen-St Clair et al., 2016).

The viral oncoprotein E6, a key factor in HPV-associated cancers, promotes p53 degradation through E6-associated ubiquitin ligase. This disruption of cell cycle checkpoints, evasion of apoptosis, and inactivation of p21 result in cell cycle arrest in the G1 phase (Pal & Kundu, 2019; Johnson et al., 2020). In contrast, non-HPV-associated head and neck cancers commonly exhibit p53 gene mutations, leading to loss of p53 function and facilitating invasion, metastasis, and cancer cell proliferation (Nathan et al., 2022). Studies

indicate that patients with HPV-positive HNSCCs lacking p53 expression due to E6-mediated degradation have a more favourable prognosis and improved overall survival (Smith et al., 2010).

E7, in contrast, exhibits strong binding to pRb and promotes its degradation through the proteasomal pathway, leading to the release of the E2F transcription factor and subsequent stimulation of cell cycle progression into the S phase (Boyer et al., 1996; Berezutskaya & Bagchi, 1997; Pal & Kundu, 2019). E7-mediated pRb degradation also leads to the upregulation of p16. The detection of p16 overexpression serves as a molecular hallmark for identifying HPV-associated OPSCCs and positively impacts patient survival in these cases. However, this association is not firmly established for non-oropharyngeal subsites of HNSCCs (Bishop et al., 2015; Du et al., 2019), where studies report either a lack of p16 expression, even in the presence of HPV mRNA, or similar levels of p16 expression regardless of HPV status (Castellsagué et al., 2016; Senga & Grose, 2021).

However, the expression of viral oncogenes E6 and E7 is necessary but not sufficient for the development of epithelial dysplasia and HPV-associated carcinomas. Through molecular analysis of cervical cancer tissues, it has been observed that the viral genome often integrates into the genome of host cells (zur Hausen, 2000).

To infer the involvement of the virus in oncogenesis, it is necessary to establish its transcriptional activity (Snijders et al., 2003; Jung et al., 2010). Transcriptionally active HPV markers traditionally include overexpression of p16, as well as the expression of E6 and E7 proteins (Wiest et al., 2002; Jung et al., 2010; Kato et al., 2020).

Overall, HPV-positive HNSCC has a better prognosis than HPV-negative HNSCC. Several studies have shown that patients with HPV-positive OPSCC respond better to treatment than patients with HPV-negative OPSCC (Kumar et al., 2007, 2008).

Detection of p16 in OPSCC has recently become a routine in Latvia. However, HPV status determination is not standard for HNSCC patients, despite widespread implementation in the developed countries.

Aim of the Thesis

This research aims to investigate the prevalence of HPV infection (HPV DNA and E6/E7 mRNA) in patients with OPSCCs, HPSCCs, and LSCCs, as well as its significance in tumour development and survival of the patients with the additional assessment of the immunohistochemical (IHC) expression of tumour suppressor proteins (p16 and p53) and HR-HPV E6 and E7 oncoproteins.

Objectives of the Thesis

The following objectives are set to reach the aim of the doctoral thesis:

1. Analyse the associations among medical history data (patient's gender and age, survival data), primary tumour location, TNM data, risk factors (smoking and alcohol consumption), morphological and molecular virology findings to investigate the role of HPVs in the development of OPSCC, HPSCC, and LSCC, as well as their impact on survival.
2. Determine the presence of HPV's DNA (low-risk [LR-] and HR-) in HNSCC tissues using PCR with consensus primers.
3. Determine the presence of HR-HPV's genomic DNA (especially HPV16, 18) in HPV+ HNSCC.
4. Determine transcriptional activity of HR-HPV in HR-HPV+ HNSCC by detecting E6/E7 mRNA.
5. Analyse the IHC expression of tumour suppressor proteins p16 and p53, as well as HPV E6/E7 oncoproteins, in tissues from patients with histologically confirmed OPSCC, HPSCC, and LSCC.

Hypotheses of the Thesis

1. HPV infection plays a role in the development of OPSCC, HPSCC, and LSCC.
2. HPV status in patients with OPSCC, HPSCC, and LSCC is an independent prognostic factor.
3. p16, p53, HR-HPV E6/E7 oncoproteins have prognostic value and impact survival.
4. There are associations between p16, p53, HPV status (HPV DNA, E6/E7 mRNA, HR-HPV E6/E7 oncoprotein immunoexpression), and survival in patients with OPSCC, HPSCC, and LSCC.

Novelty of the Thesis

The novelty of this research lies in exploration of HPV's involvement in LSCC and HPSCC aetiopathogenesis. While the role of HPV in OPSCC has been well-established, there remains significant uncertainty regarding its association with LSCC and HPSCC. Through comprehensive molecular and immunohistochemical analyses, this study confirms the participation of HPVs in the development of LSCC and HPSCC, shedding light on a previously neglected aspect of HPV-related cancers.

A key highlight of this research is the demonstration of the effectiveness of immunohistochemical detection of HR-HPV E6/E7 oncoproteins as potential prognostic markers specifically in non-OPSCC cases. The utilization of immunohistochemistry emerges as a valuable tool for evaluating the prognosis of these cancers, significantly enhancing our comprehension of HPV's role in the carcinogenesis of LSCC and HPSCC. Ultimately, this study stands as a pioneering contribution to broadening our understanding of HPV-associated HNSCC, extending beyond the established domain of OPSCC. The findings hold promise for influencing diagnostic and prognostic strategies in the context of LSCC and HPSCC.

1 Materials

The research consisted of two parts – retrospective and prospective.

The retrospective study was conducted at Riga East Clinical University Hospital Latvian Oncology Centre; 247 patients diagnosed with OPSCC were included, staged following the TNM classification of the International Union against Cancer (UICC, 6th edition) for oropharyngeal carcinoma. The study period ranged from January 1st, 2000, to December 31st, 2010. The patients' data was obtained from the Hospital Archive and The Centre for Disease Prevention and Control. The collected included patient survival status, death date (if applicable), age at the time of the diagnosis, sex, TNM status (UICC 6th edition), disease stage, hazardous habits, therapy modality, primary tumour location, and histopathological variant of the tumour.

The second part included a complex analysis of 106 patients with histologically diagnosed HNSCC. The period of patient enrolment was between January 2015 and August 2019. The research was performed by the means of gathering patients' clinical data and performing IHC and molecular analysis of the gathered tumour samples. Several morphological methods were used in the research – IHC and immunofluorescence. Additionally, different molecular biology methods were used. The study was conducted at Riga East University Hospital Latvian Oncology Centre. In addition to fresh tumour samples obtained during surgery or biopsy, paraffin-embedded, formalin-fixed tumour tissue (FFPE) blocks along with the histopathology reports were collected from the Pathology Centre of Riga East Clinical University Hospital. All morphological studies were conducted at the Joint Laboratory of Electron Microscopy, Institute of Anatomy and Anthropology. The molecular biological studies were performed at the Institute of Microbiology and Virology, Riga Stradiņš University.

2 Methods

2.1 The first part. Survival analysis of patients with OPSCC linked to histopathology, disease stage, tumour size, risk factors, and received therapy

The study is described in the manuscript "Lifsics, A., Rate, E., Ivanova, A., Tars, J., Murovska, M., and Groma, V. (2020). Survival analysis of oropharyngeal squamous cell carcinoma patients linked to histopathology, disease stage, tumor stage, risk factors, and received therapy. Experimental oncology, 42(1), 51–59. https://doi.org/10.32471/exp-oncology.2312-8852.vol-42-no-1.14147".

A retrospective study involved 247 patients with histopathologically confirmed OPSCC. The collected data were analysed to determine the overall and disease-specific 3-year and 5-year survival rates for all patients and hazard ratios of analysed variables to determine the significant factors affecting patients' survival. Patients' characteristics are summarised in Table 2.1.

FFPE samples of OPSCC were retrieved from the archival files of the Department of Pathology Oncology Centre of Latvia. Pathology reports for all tumours were reviewed, and the analysis was conducted on sections stained with haematoxylin and eosin. Tumours were classified based on their histological features. However, it should be noted that in the early years of this retrospective study, certain factors such as the pattern of invasion at the tumour edge, presence of perineural invasion, and immune system response, as proposed by Brandwein and co-authors (Brandwein-Gensler et al., 2005) and subsequently discussed by other researchers (Duvvuri et al., 2014), were underestimated. Consequently, the histopathological assessment did not consider the revision of surgical margins and the evaluation of supplemental tissue. Microphotographs were captured using Leitz DMRB bright-field optics equipped with a digital camera DC 300F.

Table 2.1

Patients' characteristics

Sex – *n* (%):	
• Male	227 (91.90)
• Female	20 (8.10)
Age (years):	
• Mean (SD)	60 (8.985)
• Range	27–85
Disease stage – *n* (%)*:	
• I	3 (1.22)
• II	19 (7.72)
• III	61 (24.80)
• IV	163 (66.26)
T stage – *n* (%)**:	
• T1	23 (9.39)
• T2	59 (24.08)
• T3	73 (29.80)
• T4	90 (36.73)
N stage– *n* (%)*:	
• N0	77 (31.30)
• N1	54 (21.95)
• N2	82 (33.33)
• N3	30 (12.20)
• Nx	3 (1.22)
Alcohol abuse – *n* (%)***:	
• Yes	82 (35.19)
• No	151 (64.81)
Smoking – *n* (%)****:	
• Yes	180 (75.95)
• No	57 (24.05)
Alcohol and smoking – n (%):	73 (31.47)

*Unknown for 1 patient
** Unknown for 2 patients
***Unknown for 14 patients
**** Unknown for 10 patients

2.2 The second part. Prospective study of 106 patients with HNSCC

The results of this part have been published in three manuscripts:

- Lifšics, A., Čistjakovs, M., Groma, V. & Murovska, M. (2021). Detection and Genotyping of Human Papillomavirus in Hypopharyngeal Carcinoma Samples. Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences., *75*(1), 11-15. https://doi.org/10.2478/prolas-2021-0002
- Lifsics, A., Groma, V., Cistjakovs, M., Skuja, S., Deksnis, R., & Murovska, M. (2021). Identification of High-Risk Human Papillomavirus DNA, p16, and E6/E7 Oncoproteins in Laryngeal and Hypopharyngeal Squamous Cell Carcinomas. *Viruses*, *13*(6), 1008. https://doi.org/10.3390/v13061008
- Lifsics, A., Cistjakovs, M., Sokolovska, L., Deksnis, R., Murovska, M., & Groma, V. (2023). The Role of the p16 and p53 Tumor Suppressor Proteins and Viral HPV16 E6 and E7 Oncoproteins in the Assessment of Survival in Patients with Head and Neck Cancers Associated with Human Papillomavirus Infections. *Cancers*, *15*(10), 2722. https://doi.org/10.3390/cancers15102722

The study was approved by the Ethical Committee of Riga Stradiņš University (Decisions No. 3/24.09.2015.) and conducted according to the Declaration of Helsinki.

2.2.1 Patients' characteristics

The sex, age, TNMG status, smoking and drinking habits, and treatment modalities were assessed for each patient. The survival data were gathered from The Centre for Disease Prevention and Control on 1 January 2022. In total, 34 of 106 patients had OPSCC, 41 had LSCC, and 31 had HPSCC. The patients' data is summarised in Table 2.2.

Table 2.2.

Patients' characteristics

	Cases (*n* = 106)		
	OPSCC (*n* = 34)	**LSCC (*n* = 41)**	**HPSCC (*n* = 31)**
Sex:			
• Male	27	39	29
• Female	7	2	2
Age (median)	58.5	64.3	65.9
T stage:			
• T1	6	4	0
• T2	6	8	4
• T3	6	24	16
• T4	16	5	11
N stage:			
• 0	1	35	6
• 1	15	4	16
• 2	12	2	8
• 3	6	0	1
M stage:			
• 0	34	40	27
• 1	0	1	4
G stage *:			
• 1	5	5	6
• 2	21	34	21
• 3	7	2	4
Hazards:			
• None	9	4	3
• Smoking	8	29	20
• Smoking and alcohol abuse	17	8	8
Treatment ˆ:			
• RT	16	1	21
• OP	0	9	0
• RT+OP	2	29	4
• RT+ChT (Cetuximab)+/−OP	10	0	0
• Symptomatic	6	1	6

* One patient had a missing value in the OPSCC group. ˆ One patient had a missing value in the LSCC group. RT – radiotherapy, OP – surgical treatment, ChT – chemotherapy.

2.2.2 Methods of molecular biology

The DNA extraction from fresh frozen tissue material was performed with the standard phenol/chloroform extraction method. DNA extraction from FFPE was performed using the blackPREP FFPE DNA Kit (Analytik Jena, Germany) following the manufacturer's protocol.

Only specimens positive for HR-HPV DNA were submitted to RNA extraction. Standard RNA extraction with TRIzol LS Reagent from Thermo Fisher Scientific was used for fresh frozen tissue specimens according to the producer's manual. A PureLink FFPE Total RNA Isolation Kit (Thermo Fisher Scientific, USA) was used for RNA extraction from FFPE cancer samples, following the manufacturer's protocol.

All extracted DNA samples were submitted for testing by the PCR with consensus primers MY9/MY11 and GP5+/6+ to detect a wide range of HR-HPV and LR-HPV types. Results were visualised by electrophoresis in 1.7 % agarose gel. Amplification products of 450 bp and 150 bp length for MY09/11 and GP5+/6+ respectively were considered HPV positive. Positive and negative controls were included in each reaction.

Type-specific primers for HPV16 and 18 were used in the PCR reaction. Amplification using HPV16 specific primers produces 152 bp long amplicons and using HPV18 specific primers – 216 bp amplicons. All available specimens were analysed using these primers.

Anyplex II HPV28 multiplex real-time PCR was performed as recommended by the manufacturer (Seegene, South Korea). The results were analysed using the Seegene Viewer software (Seegene, Seoul, Republic of Korea). Only specimens positive in consensus PCR or PCR with HPV16/18 L1 primers were analysed with this kit.

HPV High-Risk Screen Real-TM Quant (Sacace Biotechnologies, Italy) is an in vitro real-time amplification test for quantitative detection of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59. It includes a mixture of primer for

HPV groups A7, A9 (HPV16, 18, 31, 33, 35, 39, 45, 52, 58, 59), HPV group A5 (HPV51), and HPV group A6 (HPV56), and has an internal control (β-globin gene). Only specimens positive in consensus PCR or PCR with HPV16/18 L1 primers were analysed.

E6/E7 mRNA detection was conducted through real-time PCR using the PreTect HPV-Proofer kit. This assay allowed for the qualitative identification of HPV E6/E7 oncogene mRNA from HR-HPV types including 16, 18, 31, 33, and 45. Only samples positive for HR-HPV DNA were utilised for the detection of E6/E7 mRNA.

2.2.3 Immunohistochemical evaluation

Samples were processed as FFPE specimens for further analysis. The IHC assessment of HPV16 E6/E7 proteins, p53, and p16 proteins was performed according to a previously validated protocol (Skuja et al., 2018; Zake et al., 2018).

Briefly, 4–5 μm-thick FFPE tumour sections were mounted on SuperFrost Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany) and underwent a standard preparation process. They were then incubated overnight at 4 °C with specific primary antibodies. A monoclonal mouse anti-CDKN2A/p16^{INK4a} antibody (Abcam, Cambridge, UK, diluted 1:300, ab201980), a monoclonal mouse anti-p53 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, diluted 1:50, sc-47698), a monoclonal mouse anti-HPV16 E6 + HPV18 E6 antibody (Abcam, Cambridge, UK, prediluted, ab51931), and a monoclonal mouse anti-HPV16 E7 antibody (Santa Cruz Biotechnology, Inc., diluted 1:50, sc-6981) were used. The visualization of the IHC reactions was achieved using the HiDef Detection HRP Polymer system and diaminobenzidine tetrahydrochloride substrate kit (Cell Marque, Rocklin, CA, USA). Counterstaining of cell nuclei with Mayer's haematoxylin was performed, and negative controls were prepared by omitting the primary antibodies.

The positive immunoreactivity was indicated by the appearance of brown reaction products, with p53 and HPV16 E7 proteins showing nuclear staining, while p16 protein and HPV16 E6 protein exhibited nuclear and cytoplasmic staining. The cutoff for p16 immunostaining was set at 50 % positive tumour cells. The evaluation of p53 immunostaining was performed semiquantitatively. p53 overexpression was defined as p53 positivity in > 50 % of tumour cells with intensity = 2 or > 25 % of tumour cells with intensity = 3. Specimens that did not meet these criteria were considered p53-negative (p53−).

For the detection of E6 and E7 proteins, only HR-HPV-positive specimens, which all contained HPV16 DNA, were included. The semiquantitative estimation of E6 and E7 protein expression was conducted in 20 randomly selected visual fields of each sample. Two ways of interpretation were used for assessment:

- Depending on the proportion of immunopositive cells, the levels of the E6 and E7 immunoexpression were graded as negative – 0, weak – ≤ 10 %, moderate – 11–50 %, and strong – > 50 %, respectively (publication in *MDPI Viruses*).
- E6 and E7 immunoexpression levels were graded as negative if there were < 10 % immunopositive cells and positive if ≥ 10 % immunopositive cells were detected (publication in *MDPI Cancers*).

3 Statistical Analysis

3.1 The first part (retrospective)

Statistical analyses were performed using IBM SPSS to assess correlations between covariates and survival outcomes, as well as mean overall survival time after diagnosis. Differences between groups were evaluated using Pearson's chi-squared test or Fisher's exact test, depending on group size, with a significance level of $p < 0.05$. Cramer's V measured the association between nominal variables. To assess differences between nominal variables and mean survival time after diagnosis, the Kruskal-Wallis test or Mann-Whitney test was used based on the number of groups. Kaplan-Meier survival analysis estimated 3- and 5-year overall survival (OS) and disease-specific survival (DSS), with log-rank test assessing unadjusted survival rate differences ($p < 0.05$ considered significant). Hazard ratios were estimated using the Cox regression method.

3.2 The second part (prospective)

Statistical analyses using GraphPad Prism included normality tests for numerical data distribution, one-way ANOVA compared means between groups, Kruskal–Wallis or Friedman's test used for non-Gaussian data. The Mann-Whitney test or Wilcoxon test compared numerical values between two groups. Nonparametric Spearman's correlation analysis explored relationships between analysed groups. Statistical significance was set at $p < 0.05$.

Seegene results were semiquantitatively assessed and coded, while the Sacace assay's viral load was $\log_{10}$-transformed for statistical analysis. Cohen's κ test evaluated the agreement between HPV detection methods. Kaplan–Meier method conducted univariate survival analysis for OS and DSS, and the Cox regression method performed multivariate survival analysis. Statistical significance was set at $p < 0.05$.

4 Results

4.1 Retrospective survival analysis of 247 patients with OPSCC

The retrospective cohort study examined 247 patients with histologically confirmed OPSCC in different stages. The subsites within the cohort included palatine tonsils (n = 110, 44.52 %), the base of the tongue (n = 76, 30.77 %), soft palate (n = 20, 8.10 %), and posterior pharyngeal wall (n = 41, 16.60 %). Most of the patients had advanced disease stages. Most patients were male (n = 227, 91.90 %), with a median age of 60.20 years.

The female patients had a significantly longer mean survival time than males. However, there was no correlation between survival and gender, and the difference in OS between genders was not statistically significant. DSS in female patients was significantly better than in males. The patients were also divided into three age groups (< 55 years, 55–64 years, and > 65 years). While there were significantly more deceased patients in the older age subgroup, no correlation was observed between the age group and survival. Kaplan-Meier estimates indicated a decrease in survival with increasing age, but statistical significance was not reached when considering all three age groups. Pairwise comparisons revealed a statistically significant difference in survival between patients younger than 55 years and those older than 64 years ($p = 0.048$).

The study investigated the association between survival and disease stage, revealing a moderate correlation. Kaplan-Meier survival analysis showed borderline statistically significant differences ($p = 0.058$) in OS and DSS according to the disease stage. However, pairwise comparisons did not find statistically significant differences in OS and DSS between specific disease stages. Mean survival time and positive outcomes were found to decrease with a higher T stage, indicating a moderate correlation between outcome and tumour size. There were better OS and DSS for patients with smaller tumours (T1–2) compared to those with bigger tumours (T3–4).

There was no correlation between survival and N status. OS and DSS did not significantly differ based on N status. Smoking showed a moderate correlation with survival, with nonsmokers exhibiting better OS and DSS. There was no correlation between alcohol abuse and survival or mean survival time. Notably, patients who both smoked and abused alcohol had a statistically significant decline in OS and DSS (Figure 1 of Annex 2).

Tumour location did not impact mean survival time or OS. Patients with pharyngeal wall and tonsillar tumours exhibited the worst OS ($p = 0.03$) and DSS ($p = 0.026$) estimates, while those with soft palate tumours had better outcomes. Most tumours were keratinizing squamous cell carcinomas (KSCC; 70.85 %), with a smaller proportion of nonkeratinizing squamous cell carcinomas (NKSCC; 19.43 %), undifferentiated carcinomas (1.21 %), or adenosquamous carcinomas (0.4 %). The histological variant of the tumour did not significantly affect OS or DSS.

Tissue samples of KSCC showed large polygonal squamous cells with distinct cell borders, keratin formation, and a range of grades, from well-differentiated to poorly differentiated, with varying degrees of keratinization. Keratin pearls, indicative of keratin formation, were observed. Even in poorly differentiated tumours lacking keratinization, there was diffuse squamous maturation. The KSCC samples often consisted of discrete nests with abundant eosinophilic cytoplasm and nuclear pleomorphism. Infiltrative nests of tumour cells within desmoplastic stroma were common. NKSCC tumours formed nests, sheets, and cords with well-defined borders. These tumours featured relatively monomorphic, densely packed basaloid cells with ovoid and spindle-shaped morphology, indistinct cell borders, highly hyperchromatic nuclei, and a high nuclear-to-cytoplasmic ratio.

While the study did not specifically differentiate between HPV-driven tumours and HPV-negative tumours, it can be speculated that KSCC tumours are highly likely to be HPV-negative, while NKSCC tumours are suggestive of HPV

involvement. NKSCC tumours typically formed sheets, nests, and cords with sharply defined borders, and the tumour cells exhibited basaloid features with peripheral palisading.

To comprehend the aggressive nature of the tumour, factors like perineural spread, lymphovascular invasion, and muscular invasion were assessed, recognizing that histological grade based on keratinization alone might not consistently predict clinical outcomes. The study showed that perineural invasion and lymphovascular invasion were frequently present in squamous cell carcinomas, and their presence correlated with decreased survival rates. Additionally, as tumour masses invaded deeper, malignant cells infiltrated underlying skeletal muscle tissue, forming islands and cords.

A strong correlation between survival and therapy was observed. However, there was no correlation between applied therapy and mean survival time. Significant differences in OS and DSS were found among different therapeutic modalities. Patients in the OP and RT+OP groups had better survival outcomes compared to other treatment groups. Pairwise comparisons revealed significant differences in OS only between the RT and RT+OP groups, RT+OP and RT+ChT (Cetuximab) +/−OP groups ($p < 0.05$), and borderline significance between the RT and OP groups. These findings suggest higher survival rates for surgical interventions, while the RT group (excluding symptomatic treatment) had the lowest survival rates (OS and DSS).

Among the patients included in the study, the majority did not receive surgical intervention as part of their treatment (n = 196). Only a small number of patients underwent primary tumour excision (n = 10), neck dissection (n = 28), or both (n = 13). Analysing the impact of surgical intervention on patient outcomes, we found that the number of deceased patients was significantly higher when no operation was performed.

Additionally, the mean OS time after the diagnosis of the disease was significantly longer in surgically treated patients. However, we did not find a

correlation between mean OS time and the specific type of surgery performed. Kaplan-Meier analysis demonstrated significant differences in survival (OS and DS) depending on whether the patient underwent surgery or not, with significantly higher survival rates in patients who underwent surgery ($p < 0.0001$). Nevertheless, pairwise comparisons of different types of surgical procedures did not show any significant differences in OS ($p = 0.29$) or DS ($p = 0.11$).

Multivariate Cox regression analysis showed that the T2 stage, N status, the presence of smoking or alcohol abuse, and the treatment modality of RT+OP had a statistically significant impact on the risk of death. Patients with T2 tumours had a 57 % and 77 % lower risk of early death compared to patients with T3 and T4 tumours, respectively. Furthermore, N0 status was associated with a 34 % lower risk of early death compared to N+ status.

The study also revealed that the combination of OP and RT as a treatment modality had a significantly lower risk of early death compared to other treatment modalities, including RT alone or in combination with ChT with cetuximab (Figure 2 of Annex 2).

The risk of early death was 300 % lower for RT+OP compared to symptomatic treatment, and 154 % lower compared to RT+ChT (Cetuximab)+/−OP. Additionally, when comparing the combination of RT+OP to RT or OP alone, the hazard of death was estimated to be 2.02 and 1.27 times higher for RT and OP, respectively. The Cox regression multivariate analysis further confirmed that alcohol abuse and/or smoking significantly increased the risk of early death.

4.2 The first publication of the prospective part

4.2.1 Detection of HPV genomic sequences in HNSCC samples (FFPE tissue blocks)

DNA extraction from FFPE tissue blocks using the *black PREP FFPE DNA Kit* was a relatively easy and fast procedure. The lowest extracted DNA concentration was 16.54 ng/µl, in most of the extracted DNA samples the concentration was above 60 ng/µl. All extracted DNA samples were β-globin positive, which made them viable for further analysis.

Sample screening by MY09/11 consensus primers detected only 1/31 positive sample. However, PCR using GP5+/6+ consensus primers was much more proficient, resulting in 100 % positivity (n = 31) for HPV DNA.

HPV genotyping using type-specific primers (HPV16 and 18) showed positivity for HPV16 only – 15/31 (48.4 %).

The HPV detection rate using the Anyplex II HPV28 assay was 14/31 (45.2 %). In one case, there was a coinfection of two HPV types (type 16 and 56). The remaining 13 cases had HPV16 monoinfection.

The HPV detection rate using Sacace HPV High-Risk Screen Real-TM Quant was 12/31 (38.7 %) in the HEX channel only, which corresponds to the HPV A9 group (16, 31, 33, 35, 52, 58).

4.2.2 Comparison of genotyping results obtained by different detection systems

The same DNA extracts from the 31 selected FFPE samples tested by consensus primers and HPV16 specific primers, were further subjected to Anyplex II HPV28 assay, and Sacace HPV High-Risk Screen Real-TM Quant assay. Valid results with the use of both assays were obtained for all 31 biopsy samples.

There were many discordant results between PCR with HPV16 specific primers and real-time PCR assays (Anyplex and Sacace). The agreement of PCR

with HPV16 specific primers and Anyplex assay could not be assessed because of the high p-value of Cohen's kappa (Cohen's κ coefficient = 0.288, $p = 0.156$). A comparison of the results from PCR with HPV16 specific primers and Sacace assay showed a similar result (Cohen's κ coefficient = 0.285, $p = 0.149$), meaning the agreement between these two methods could not be assessed with significance.

Among the 14 HPV-positive samples by the Anyplex assay, 11 (78.6 %) were found positive by the Sacace assay. The agreement between both methods was good (Cohen's κ coefficient = 0.736, $p < 0.001$)

Additionally, there was a moderate positive correlation between viral load (assessed by Sacace assay) and semiquantitative Seegene assay results estimated semiquantitatively ($r_S = 0.60$, CI 0.30-0.79, $p = 0.0004$; Figure 3 of Annex 2).

4.3 The second publication of the prospective part

4.3.1 Presence of HPV genomic sequences in tumour samples (HPSCC and LSCC)

Out of the 72 tumour samples tested, 11 samples (15.3 %) were positive for HPV genomic sequences using MY09/11 primers, while 55 samples (76.4 %) showed positivity using GP5+/6+ primers. Overall, when tested with consensus PCRs, 61 tumour tissue samples (84.7 %) were found positive for HPV DNA, with 31 samples identified as HPSCC and 30 samples as LSCC.

4.3.2 HPV genotyping using HPV16 and HPV18 L1 primers and Anyplex II HPV28 real-time PCR

All 72 tumour tissue samples were subjected to HPV genotyping using HPV16 and HPV18 L1 primers. Two tumour samples (both LSCC) that were positive when detected by HPV16 L1 primers were negative in consensus PCRs. No specific HPV18 genomic sequence was found in any of the samples. In total, 26 samples (36.1 %) were positive for HPV16, with 10 samples identified as

LSCC and 16 as HPSCC. A total of 63 HPV DNA+ samples were applicable for further analysis.

The 63 HPV-positive samples confirmed using consensus primers or HPV16-specific primers were further analysed using the Anyplex II HPV28 multiplex real-time PCR. Out of the 63 samples, 28 samples were HPV-negative when assessed by the Anyplex II HPV28 assay. Among the remaining HPV-positive samples, 32 samples showed HPV16 monoinfection, 2 samples showed HPV16 and HPV31 coinfection, and 1 sample showed HPV16 and HPV56 coinfection. Among 32 HPV+ samples, 19 LSCC and 13 HPSCC samples were HPV16+, 2 LSCC samples showed HPV16 and HPV31 coinfection, and 1 HPSCC sample showed HPV16 and HPV56 coinfection.

Interestingly, 7 tumour tissue samples (1 LSCC and 6 HPSCC) initially confirmed as HPV16+ using HPV16 L1 primers' PCR tested negative using Anyplex II HPV28 real-time PCR, contributing to a total of 42/72 (58.3 %) HPV16+ samples. The prevalence of HPV16 infection, including multiple infections in a sample, was 22/41 (53.7 %) for LSCC and 20/31 (64.5 %) for HPSCC. All HPV16+ HPSCC samples were stage III or IV tumours (Figure 4 A of Annex 2).

Among the HPV16+ samples, 21 samples showed low viral load, 9 samples showed moderate viral load, and 2 samples showed high viral load using the Anyplex II assay (Figure 4 B of Annex 2).

4.3.3 Expression of p16 detected by IHC

IHC analysis confirmed that 11.1 % of the tumour tissue samples exhibited expression of p16. Among the 41 samples of LSCC and 31 samples of HPSCC, six and two samples, respectively, showed positive p16 expression. By comparing p16 and HPV status, the tumours were categorised as follows: 7/72 (9.7 %) were p16+/HPV+, 1/72 was p16+/HPV−, 8/72 (11.1 %) were p16−/HPV−, and 56/72 (77.8 %) were p16−/HPV+. The majority of p16+/HPV+

tumours were LSCC (5 cases), while two cases were HPSCC. There was only one case of p16+/HPV− tumour, which was LSCC. Among the seven p16+/HPV+ tumours, six had HPV16 as the sole infection, while one case had co-infections of HPV16 and HPV31. Among the 56 p16−/HPV+ tumours, 27 were LSCC and 29 were HPSCC. Out of these, 35 showed HPV16 monoinfection when examined using Anyplex II real-time PCR and HPV16 L1 primers' PCR, whereas two cases had the mentioned HR-HPV co-infections (Figure 4 C of Annex 2).

4.3.4 Expression of HPV16 E6 and E7 oncoproteins detected by IHC

IHC detection of HPV16 oncoproteins E6 and E7 was performed in 42 FFPE samples (22 LSCC and 20 HPSCC). The detection was based on the primary recognition of HPV16 as the main HPV type using molecular virology assays.

Expression of E6 oncoprotein in HPV16+ LSCC specimens was detected in 21/22 cases. The immunoreactive structures were observed within the tumour mass and the surface epithelium, showing dysplastic features. In some cases, only the tumour nest contained the E6 oncoprotein. Strong immunoexpression of HPV16 E6 oncoprotein in the tumour mass (> 50 %) was observed in 3/22 cases, and two of them also showed strong positivity in the dysplastic epithelium. (Figure 1 A of Annex 1; Figure 5 A, B of Annex 2). 12/22 LSCC samples showed low expression of E6 oncoprotein in the tumour mass (Figure 5 A of Annex 2). In the dysplastic epithelium, the distribution of E6 expression levels varied. Three cases showed E6-negative dysplastic epithelial cells, in two of them there was low immunopositivity in the tumour mass (Figure 5 B of Annex 2). In most specimens, positive staining in the invasive front was noticed, commonly in the suprabasal cells (Figure 1 B, C of Annex 1). HPV16 E6 viral protein expression

was also frequently observed in the endothelial cells of small blood vessels (Figure 1 B, C of Annex 1).

HPV16 E7 protein immunoexpression was confirmed in 20/21 LSCC specimens (Figure 5 C of Annex 2). The labelled cells displayed nuclear staining, with some showing nuclear and cytoplasmic staining. The expression of HPV16 E7 oncoprotein was observed in pseudostratified ciliated epithelium and stratified squamous epithelium, predominantly in basal and suprabasal cells (Figure 2 A, B of Annex 1).

Strong immunoexpression of HPV16 E7 oncoprotein in the tumour nests was found in 8 out of 21 LSCC samples (Figure 2 C of Annex 1; Figure 5 C, D of Annex 2).

In HPSCC samples, 18/20 were positive for HPV16 E6 oncoprotein (Figure 5 E, F of Annex 2). Most of the samples showed detectable levels of HPV16 E6 oncoprotein within the cytoplasm of dysplastic epithelial cells. The expression of E6 oncoprotein within the tumour mass was generally low (Figure 1 D of Annex 1).

13/20 HPSCC cases showed positivity for HPV16 E7 oncoprotein, primarily in the nucleus (Figure 5 G, H of Annex 2). Positive reactions were observed in the tumour mass and suprabasal cells, as well as in endothelial cells (Figure 2 D of Annex 1).

Overall, there were no significant differences in tumoral or dysplastic epithelial HPV16 E6/E7 oncoprotein expression, except for a significant difference in E6 oncoprotein positivity in HPSCC samples (Figure 5 E of Annex 2). In general, a comparable pattern of HPV16 E6/E7 oncoprotein expression was observed within both the tumour mass and dysplastic epithelium in both LSCC and HPSCC, as illustrated in Figures 5 A, C, and G of Annex 2.

Semiquantitative real-time PCR and E6/E7 oncoprotein immunoexpression results were subjected to nonparametric correlation analysis. A moderate positive correlation ($r_S = 0.445$, $p = 0.056$) was observed between

semiquantitative real-time PCR and HPV16 E7 IHC data in LSCC tissue samples, particularly in the dysplastic epithelium. Weak to moderate positive correlations were found in HPSCC tissue samples, but they did not reach statistical significance.

4.4 The third publication of the prospective part

4.4.1 HPV DNA and genotypes in different types of HNSCC

Samples from patients with HNSCC were analysed to determine the presence of HPV DNA and its genotypes. 92/106 (86.79 %) HNSCC samples were HPV DNA-positive. The presence of HPV DNA varied across different types of HNSCC: 29/34 OPSCC samples (85.29 %), 32/41 LSCC samples (78.05 %), and all 31 HPSCC samples (100 %) were positive for HPV DNA. The most common HR-HPV genotype detected was HPV16, which was found in 68/106 (65.09 %) of HNSCC samples. HPV16 was prevalent in 26/34 (76.47 %) OPSCC samples, 22/41 (53.66 %) LSCC samples, and 20/31 (64.52 %) HPSCC samples. HPV coinfections with HPV16 were observed in 7/106 HNSCC samples, with HPV31 detected in 2 samples, HPV33 in 1 sample, HPV35 in 1 sample, and HPV56 in 4 samples. Given its high prevalence, further analysis focused on HPV16.

4.4.2 HPV16 E6/E7 mRNA in HPV16-positive HNSCC sample

The study analysed the presence of HPV16 E6/E7 mRNA in HPV16-positive HNSCC samples. Among the HPV16-positive samples, HPV16 E6/E7 mRNA was detected in 15/26 (57.7 %) OPSCC samples, 2/22 (9 %) LSCC samples, and 0/20 HPSCC samples. A correlation analysis revealed a moderate positive correlation between the semiquantitative HPV16 viral load and the presence of HPV16 E6/E7 mRNA ($r_S = 0.601$, $p < 0.0001$). Additionally, a weak positive correlation was observed between p16 overexpression and E6/E7

mRNA expression (r_S = 0.472, p < 0.0001). However, no correlation was found between p53 downregulation (p53−) and E6/E7 mRNA expression.

4.4.3 Expression of p16 in HNSCC samples detected by IHC

IHC was performed to assess the overexpression of p16 in HNSCC samples (Figure 3 A of Annex 1). Among the 106 HNSCC samples, p16 overexpression was observed in 24 samples (22.64 %). Specifically, it was found in 16/34 OPSCC samples (47.06 %), 6/41 LSCC samples (14.63 %), and 2/31 HPSCC samples (6.45 %). When considering HPV16 positivity, p16 overexpression was confirmed in 15/26 HPV16+ OPSCC samples (57.69 %), 5/22 HPV16+ LSCC samples (22.73 %), and 2/20 HPV16+ HPSCC samples (10 %).

4.4.4 Expression of p53 in HNSCC samples detected by IHC

The study revealed that p53 overexpression (p53+; Figure 3 B of Annex 1) was confirmed in 49/106 (46.23 %) HNSCC samples. Among the different subtypes of HNSCC, p53 overexpression was observed in 17/34 (50 %) OPSCC samples, 21/41 (51.22 %) LSCC samples, and 11/31 (35.48 %) HPSCC samples.

Subsequently, an analysis of the HPV16+ samples showed p53 downregulation in a significant proportion of cases. 15/26 (57.69 %) OPSCC samples were p53−, while 10/22 (45.45 %) LSCC samples were p53−. In the case of HPSCC samples, 14/20 (70 %) were p53−.

Furthermore, in the subset of samples positive for E6/E7 mRNA, which indicates the presence of active HPV infection, p53 downregulation was found in 11/15 (73.33 %) OPSCC samples and 1/2 (50 %) LSCC samples.

4.4.5 Expression of HPV16 E6 and E7 oncoproteins in HNSCC samples detected by IHC

Overexpression of HPV16 E6 protein (Figure 3 C of Annex 1) was confirmed in 44/106 (41.5 %) HNSCC samples. Specifically, it was observed in

21/34 (61.8 %) OPSCC samples, 14/41 (34.1 %) LSCC samples, and 9/31 (29.0 %) HPSCC samples.

Similarly, overexpression of HPV16 E7 protein (Figure 3 D of Annex 1) was found in 39/106 (36.8 %) HNSCC samples. Specifically, it was observed in 19/34 (55.9 %) OPSCC samples, 14/41 (24.1 %) LSCC samples, and 6/31 (19.4 %) HPSCC samples.

4.4.6 Kaplan–Meier survival analysis

OS and DSS depending on HPV DNA (HR-HPV and LR-HPV)

The five-year OS and DSS were assessed in HPV+ and HPV− patients based on the primary tumour location. For patients with OPSCC, the OS rates were 26.82 % for HPV+ patients and 0 % for HPV− patients, although the difference did not reach statistical significance ($p = 0.077$; Figure 6 A of Annex 2). However, the DSS rates were 27.78 % for HPV+ patients and 0 % for HPV− patients, with statistical significance ($p < 0.05$; Figure 6 B of Annex 2).

For patients with LSCC, the OS rates were 64.59 % for HPV+ patients and 44.44 % for HPV− patients, demonstrating statistical significance ($p < 0.05$; Figure 6 C of Annex 2). The DSS rates were 68.90 % for HPV+ patients and 50 % for HPV− patients, also showing statistical significance ($p < 0.05$; Figure 6 D of Annex 2).

Due to all HPSCC samples being HPV DNA-positive, a Kaplan-Meier survival analysis could not be performed for this group.

OS and DSS depending on HPV16 DNA, HPV16 E6/E7 mRNA, and IHC expression of p16, p53, E6, and E7 proteins

A Kaplan-Meier survival analysis was conducted, stratifying patients based on the primary tumour location. The OS and DSS were calculated, and for most variables, the univariate survival analysis using the Kaplan-Meier method did not reach statistical significance.

However, there were borderline statistically significant differences ($p = 0.057$; Figure 7 A, B of Annex 2) in OS and statistically significant differences in DSS between p16+ and p16− OPSCC patients.

The analysis of p53+ and p53− HPSCC patients showed statistically significant differences in OS and DSS (Figure 7 C, D of Annex 2) with significantly better survival for the p53− group.

IHC overexpression of HPV16 E6 protein was associated with significantly better OS and DSS in patients with OPSCC (Figure 7 E, F of Annex 2).

4.4.7 Multivariate Cox regression analysis

OPSCC

The group consisted of 34 patients, of which 26 died. Two patients were excluded from the analysis due to missing values. The Cox regression analysis revealed that the IHC expression of p16, p53, and HPV16 proteins E6 and E7, the tumour size, the applied treatment, and smoking significantly affected the survival of OPSCC patients.

The overexpression of p16, p53, and HPV16 E6 protein were associated with much lower hazard ratios, indicating a significantly improved survival outcome. On the other hand, the overexpression of HPV16 E7 protein was associated with a higher risk of early death.

A graphical analysis further supported these findings. Patients with p16+ tumours had better survival outcomes compared to patients with p16− tumours (Figure 8 A of Annex 2). However, the overexpression of HPV16 E7 protein was associated with decreased survival. Interestingly, when combining the two markers (p16 and HPV16 E7 protein), the overexpression of E7 protein led to decreased survival, even in patients with p16+ tumours (Figure 8 A of Annex 2).

Another interesting finding was related to the IHC expression of p53 and HPV16 E6 protein. Patients with p53+/E6+ tumours had the best survival outcomes, while those with p53−/E6− tumours had the worst survival

(Figure 8 B of Annex 2). There was no difference in survival between patients with p53−/E6+ and p53+/E6− tumours.

LSCC

The Cox regression analysis for LSCC did not show any variables significantly affecting survival.

HPSCC

The group consisted of 31 patients, of which 29 died. The Cox regression model indicated that several factors statistically significantly affected the survival of HPSCC patients. These factors included the IHC expression of p16 and HPV16 E6 protein, the presence of HPV16 DNA, the hazards, and the T, N, and M statuses.

The IHC overexpression of p16 and HPV16 E6 protein was associated with an extremely low risk of early death (Figure 9 A, C of Annex 2). However, when examining the combined status of p16 and HPV16 E7 protein, it was found that E7 protein expression did not have a significant impact on survival (Figure 9 B of Annex 2). Nevertheless, when considering the combined status of p53 and HPV16 E6 protein, it was observed that patients with E6+ tumours had better survival, and the overexpression of p53 seemed to further enhance survival in these patients (Figure 9 D of Annex 2). The group of patients with p53−/E6− tumours had the worst survival outcomes.

The presence of HPV16 DNA was associated with a significantly higher early death risk (Figure 9 E of Annex 2). Additionally, the Cox regression analysis revealed that larger primary tumours were associated with a higher risk of early death. Specifically, patients with T3 tumours had an 87 % lower risk of early death compared to patients with T4 tumours. Additionally, a lower N stage was associated with lower hazard ratios. The presence of distal metastases was found to be strongly associated with a 22-fold increase in the risk of death. Lastly, it was noted that smoking patients had a 57-fold increase in the risk of early death compared to non-smokers / non-drinkers.

5 Discussion

5.1 Lower T and N stages, absence of bad habits, and surgery result in better survival rates and lower hazard ratios in OPSCC (retrospective study)

Survival analysis was conducted on patients with OPSCC treated at a single hospital in Latvia over 10 years. The study aimed to identify prognostic factors by examining disease stage, tumour size, presence of locoregional metastases, age, sex, habits (smoking, alcohol abuse), histopathological tumour variant, primary tumour location, and received therapy. The analysis revealed that most patients were smokers (76 %) and a significant portion had drinking problems (35 %). Smoking and alcohol abuse were independently associated with decreased OS and DSS, with smoking having a more pronounced effect on DSS. Combining these risk factors further decreased survival. Similar findings have been reported in previous studies (Kuper et al., 2002; Farsi et al., 2017). A multivariate analysis using the Cox hazard model demonstrated a higher risk of early death when at least one of these risk factors was present.

The findings of the study indicated that most patients were diagnosed with advanced stages of the disease (stages III and IV), leading to a less favourable prognosis. Kaplan-Meier estimates of OS and DSS based on disease stage demonstrated poorer survival rates for patients with late-stage disease. Out of the 247 subjects included in the study, only 3 and 19 patients were diagnosed with stage I and stage II diseases, respectively. These results underscore the significance of early cancer detection and prompt referral to specialists, a notion that has been emphasised in previous research (Pitchers & Martin, 2006).

OPSCC is known for its aggressiveness, often diagnosed at advanced stages and showing a high rate of lymphatic metastasis (Yuan et al., 2018). In this study, most patients had clinically positive neck disease. While patients with positive lymph nodes had a higher risk of early death (multivariate Cox regression analysis), there were no significant differences in OS and DSS.

The study revealed a correlation between lower T categories and improved disease outcomes. This finding was supported by Kaplan-Meier estimates of OS and DSS, which indicated a significant decrease in survival as the T stage increased, with the longest survival observed in cases with lower T. However, it is important to note that the survival estimates obtained in this study were lower compared to those reported in the western hemisphere (Gillison et al., 2019).

Tumours of the pharyngeal wall and palatine tonsils were associated with the worst OS and DSS outcomes, consistent with previous research (Cohan et al., 2009). Most patients in this study had squamous cell carcinoma of the palatine tonsils and the base of the tongue.

Surgical treatment, particularly in the RT+OP group, demonstrated superior OS and DSS estimates compared to other modalities. While no significant differences were noted in survival based on the specific type of surgery, significant disparities arose when any surgical intervention was compared to no surgery. It is important to note the study's limitations, such as unequal and relatively small patient numbers. Existing literature supports surgical treatment as the essential and preferred approach for most patients (Ling et al., 2013).

Moreover, several other studies have indicated a survival advantage in patients who underwent surgical treatment, even when considering their HPV status (Karatzanis et al., 2012; Kamran et al., 2018). However, the interpretation of results concerning the impact of HPV status on survival has been a subject of controversy. Münscher et al. conducted a study that suggested that HPV status may not have a significant influence on survival (Münscher et al., 2017). Further research is needed to evaluate the outcome of OPSCC in patients undergoing unilateral or bilateral neck dissection. Nevertheless, certain studies have reported no significant disparity in long-term survival between unilateral and bilateral

neck dissection in patients with a clinically negative neck on the contralateral side (Lanzer et al., 2012; Al-Mamgani et al., 2017).

Comparing different treatment regimens, a study by Gillison et al. demonstrated the superiority of cisplatin plus radiotherapy over cetuximab plus radiotherapy in HPV-positive OSCC (Gillison et al., 2019). However, cetuximab was the only chemotherapeutic agent used for treating HNSCC in Latvia at the time of the study. Reconsidering the chemoradiotherapy regimen is warranted. Additionally, in this study, a survival analysis of patients with OPSCC revealed that younger patients had a reduced risk of early death compared to their older counterparts. It has been noted that RT can have a prolonged suppressive effect on the immune system, thereby potentially rendering certain OPSCC patients more vulnerable to tumour recurrence and poorer survival outcomes (Dovšak et al., 2018).

Prognostic factors are crucial in selecting treatment for OPSCC patients. Tumour size, the "RT+OP" therapeutic modality, hazardous habits (smoking, alcohol abuse), and locoregional lymph node metastases strongly predict patient outcomes. Neck dissection, especially ipsilateral elective neck dissection in clinically negative necks, appears necessary based on other studies (Fasunla et al., 2011; Psychogios et al., 2013). Unfortunately, this study lacked data on the patients' HPV status, limiting the evaluation of HPV's prognostic significance, a factor recognised by other researchers. The inclusion of HPV status could have provided valuable insights into patient outcomes (Andrews et al., 2009; Gillison et al., 2019).

The incidence of OPSCC has increased in recent decades, possibly due to the contributory role of HPV. HPV-positive OPSCC has a better prognosis than HPV-negative OPSCC, highlighting the importance of determining HPV status for prognostic purposes and treatment planning (Sinha et al., 2018).

The study is limited by its retrospective design and a relatively small population. Assessing the importance of treatment modalities is challenging due

to potential selection biases, such as patients with advanced cancer and poor general health receiving radiation therapy alone. The study also suggests the need for diverse chemotherapeutic interventions beyond cetuximab alone. While previous studies support supraomohyoid neck dissection as the primary treatment for clinically N0 tumours (Süslü et al., 2013), the study did not evaluate the difference between neck dissection levels and types (uni- vs bilateral).

5.2 DNA extraction from FFPE tissue blocks is reliable. Using multiple PCR assays is preferred (the first publication of the prospective part)

DNA extraction from FFPE tissue blocks and its use for testing has become more common in recent years. Moreover, the utilization of the same DNA extracts for all methods used ensures the high accuracy and applicability of the results when assessing the agreement between various HPV detection methods.

The Anyplex II HPV28 assay is an appropriate and dependable HPV detection method (Veyer et al., 2018; Baasland et al., 2019). However, there has been data acknowledging the need for additional conformational HPV16 genotype-specific molecular assay, especially for HPV-negative samples (Veyer et al., 2018). This study could not surely conclude agreement/disagreement between the Anyplex II assay and HPV16-specific primer's PCR results. There were multiple HPV16 positive samples by HPV16 specific primers' PCR, diagnosed as negative in the Anyplex II HPV28 assay and *vice versa*. It suggests the need for multiple detection methods for FFPE DNA extracts.

The genetic material extracted from FFPE is highly variable in terms of DNA quality and quantity (Lillsunde Larsson et al., 2015). There are various factors affecting the results of assessment – reagents used in a fixation procedure, the amount of tissue submitted to fixation, further tissue processing, etc. (Ludyga et al., 2012). Biopsy material from hypopharyngeal cancers is often limited due

to biopsies performed with local anaesthetic and indirect visualization. However, our results demonstrate successful HPV DNA detection even with small DNA concentrations.

The 100 % positivity by GP5+/6+ consensus primers (150bp) in contrast to 1/31 positivity by MY09/11 consensus primers (450bp) shows that primers which produce shorter amplicons are more beneficial, especially in fragmented DNA extracted from FFPE samples.

Our observations demonstrate that Anyplex II HPV28 and Sacace HPV High-Risk Screen Real-TM Quant assays could be used in a clinical laboratory to detect and genotype HPV in FFPE samples. The combination of these two assays has a beneficial effect when detecting different HPV types and assessing the viral load.

5.3 HPV may play a significant role in non-OPSCC (the second publication of the prospective part)

Available data indicates that around 20 % of LSCC and 5 % of HPSCC cases in the USA result from HPV infection (Saraiya et al., 2015). The incidence of HPV-positive head and neck cancer is generally lower in Europe (Ndiaye et al., 2014), though higher in developed countries like the United Kingdom, Denmark, and Germany compared to less developed Eastern European countries (Chaturvedi et al., 2013; Wittekindt et al., 2019). These differences are attributed to variations in lifestyles, preferences, sexual habits, and, importantly, the lack of appropriate HPV testing. Despite smoking being a significant factor in head and neck cancer development in Latvian society (Lifsics et al., 2020), this study suggests HPV's role in the carcinogenesis of non-oropharyngeal cancer, particularly with HPV16 being the predominant type observed in LSCC and HPSCC, aligning with findings from other studies (Ndiaye et al., 2014; Janecka-Widła et al., 2020).

This study highlights the high incidence of HPV-positive tumours and the involvement of HR-HPV in HPSCC and LSCC in Latvia, compared to Europe and North America. The study reveals a higher prevalence of HPV16 in both LSCC and HPSCC. However, further investigation is needed to determine the transcriptional activity of HPV infection in tumour tissue (Jung et al., 2010). Detecting HPV E6/E7 mRNA in LSCC and HPSCC tissue samples could provide additional clarity (Wittekindt et al., 2018). Distinguishing primary tumours from those that have spread from different sites, such as the oropharynx, remains a challenge, particularly in late-stage disease. Optimizing diagnostic accuracy, especially in advanced malignancy stages, is crucial. Despite the difficulty in accurately identifying the primary tumour site, there is evidence suggesting a higher prevalence of HR-HPV infection in late-stage hypopharyngeal cancer (Ernoux-Neufcoeur et al., 2011). In this study, most patients presented with stage III and IV tumours, and all HPV-positive HPSCCs were diagnosed as stage III or IV tumours.

Few studies have explored the presence of HPV oncoproteins E6 and E7 in tumour and dysplastic epithelial cells using IHC (Rodrigues et al., 2016; Brand et al., 2018). Some studies have reported HPV DNA and RNA in situ hybridization results using FFPE samples and conventional light microscopy (Augustin et al., 2020; Chi et al., 2020). This study utilised FFPE samples from HPV16-positive tumours (n = 42) identified by molecular biology methods. Most HPV16-positive samples exhibited positivity for either the E6 or E7 oncoproteins. However, the absence of E6/E7 immunostaining in some samples suggests the involvement of other non-HPV-related mechanisms in tumour development.

This study aimed to characterise tumorigenesis in the larynx and hypopharynx, focusing on HR-HPV DNA, p16, and E6/E7 oncoproteins assessed through molecular virology and IHC. While some correlations lacked statistical significance, weak to moderate positive correlations

between molecular virology and IHC results may indicate active HPV infection. However, definitive conclusions about HPV activity require further investigation. PCR confirmed HPV's DNA presence in the LSCC and HPSCC samples, but the methods used could not distinguish active from latent infections. Nevertheless, the presence of HR-HPV E6/E7 proteins, known contributors to tumour development, suggests the active involvement of HR-HPV in tumorigenesis.

In some HPV16-positive specimens, tumour cells were negative for HPV16 E6/E7 oncoproteins, while dysplastic epithelium showed positivity. Additionally, some endothelial cells were positive for HPV16 E6/E7 proteins, revealing PCR assay limitations in specifying the source of genetic material. The presence of HR-HPV E6 and E7 oncoproteins suggests potential cancerous transformation, but viral integration (Münger et al., 2004), a common mechanism in HPV-related cancers, occurs less frequently in HNSCCs. In these tumours, dysregulation of E6/E7 genes in an episomal state, possibly due to methylation disrupting HPV E2 binding sites, may occur (McBride & Warburton, 2017). The absence of HPV16 E6/E7 oncoproteins in tumour cells, coupled with their presence in dysplastic epithelial and endothelial cells, may indicate the absence of HPV integration. In advanced tumour stages, viral DNA clearance and alternative tumorigenic mechanisms may occur.

This study identified a significant number of p16−/HPV+ specimens in LSCC and HPSCC patients, suggesting that p16 may not be a practical surrogate marker of HPV infection in these cancers (Lewis et al., 2017). However, some authors propose that HR-HPV infection may contribute to laryngeal carcinogenesis through viral DNA integration into the host cell genome, leading to increased p16 expression (Torrente et al., 2011).

This study employed a comprehensive range of HPV-specific tests, including PCR, p16 and E6/E7 oncoprotein IHC. Despite strengths, limitations include moderate sample size, the absence of HPV mRNA data, and observed

gender and tumour stage imbalances, though these did not significantly impact the overall results.

5.4 HPV infection significantly impacts survival in both OPSCC and non-OPSCC patients. IHC detection of HR-HPV E6 protein serves as a convenient prognostic factor in HNSCC (the third publication of the prospective part)

The present study aimed to assess the impact of HPV infection and related markers, including p16, p53, HPV16 E6/E7 oncoproteins, the presence of HPV DNA, and E6/E7 mRNA, on patient survival. Kaplan-Meier survival analysis revealed that not only HR-HPV, but also LR-HPV infection may play a role in the survival of patients with OPSCC and LSCC. Approximately one-third of the patients had a likelihood of LR-HPV infection. The study findings indicate that patients with HPV DNA-positive OPSCC and LSCC exhibit improved 5-year OS and DSS. These results align with studies demonstrating better survival rates for patients with HNSCC and tonsillar cancer when their tumours tested positive for HPV DNA (Fakhry et al., 2008; Attner et al., 2012). A possible explanation for this observation is that HPV-positive tumours demonstrate enhanced sensitivity to radiation therapy, allowing for less aggressive treatment and better outcomes for patients (Attner et al., 2012). Additionally, HPV-infected cells might be more readily recognised by the immune system, facilitating their identification and destruction.

Studies consistently show higher 3- and 5-year survival rates in HPV-positive OPSCC compared to HPV-negative cases (You et al., 2019). While this consensus is mainly observed for HR-HPV types, particularly HPV16 and 18, several studies suggested no significant survival improvement for HPV-positive LSCC tumours (Ahmadi et al., 2018; Hughes et al., 2019). However, recent data, including this study, reveal better survival outcomes for patients with HPV-positive LSCC (H. Wang et al., 2019).

Conversely, this study focused on stratifying patients with HNSCC based on tumour location and identifying specific HPV types. We discovered that the presence of HPV16 DNA in HPSCC cases markedly decreased patient survival rates, suggesting a significant role of HPV16 in HPSCC development. However, the immunological aspects should be taken into consideration. The presence of viral antigens could potentially stimulate anti-tumour immune responses, leading to improved patient survival (Masterson et al., 2016; Cillo et al., 2020).

This study reconfirmed p16's predictive role in OPSCC through univariate survival analysis, aligning with prior research (Wendt et al., 2021). Cox regression analysis emphasised p16 as a distinct predictive marker for OPSCC, with statistical significance. While the univariate analysis of HPSCC and LSCC did not confirm this association, Cox regression suggested better survival and reduced risk of death in p16+ HPSCCs, hinting at its potential as a predictive marker. This aligns with findings from other studies (Tribius et al., 2018; Shi et al., 2022). The association between p16 and HPV activity in non-OPSCC raises questions about its use as a surrogate marker for HPV infection and its suitability for survival prognosis. Studies indicate that p16 often does not correspond to the HPV status in non-oropharyngeal cancers, but it does have prognostic value for survival (Sánchez Barrueco et al., 2019; Gallus et al., 2022).

The univariate survival analysis of p53 IHC expression showed significantly better OS and DSS for the patients with p53-negative HPSCC, which could be attributed to the suppressing function of the E6 protein of HR-HPV, contrary to the Cox regression analysis, without a statistical significance, however. Cox regression analysis of OPSCC patients showed that p53 overexpression was associated with a significantly lower risk of death. This observation could be attributed to the tumour-suppressing properties of p53. However, there was a substantial number of HPV16+ samples, including samples positive for HPV16 E6/E7 mRNA in OPSCC. In HPV-driven cancers, it is logical to expect p53 suppression, resulting in a p53− result. Published data

suggest that HPV-driven tumours exhibit p53 downregulation (S. Wang et al., 2021). Conversely, several studies reported that p53 overexpression correlates with a better response to chemotherapy and is associated with improved survival (Hasegawa et al., 2018; Sun et al., 2021). However, these studies did not investigate HPV status. Initially, in HPV-driven cancers, there could be p53 overexpression due to the degradation of pRb by the E7 oncoprotein, leading to increased stabilization of p53 (Howie et al., 2009). A meta-analysis of tongue squamous cell carcinoma indicated that p53 could not be used as a prognostic biomarker for these tumours (Almangush et al., 2017). Similar conclusions were drawn by Halec et al. for LSCC (Halec et al., 2013). Unfortunately, our study did not assess TP53 gene mutations, which could have provided clarity on the aforementioned issues (Zhou et al., 2016). Additionally, there is a possibility that p53 overexpression is unrelated to HPV infection, particularly considering the high number of smokers in our study. Further studies are needed to explore the prognostic role of p53 in HNSCC, especially in OPSCC and HPSCC.

To our knowledge, there have been limited studies investigating the IHC expression of HPV oncoproteins E6/E7 and their role in survival or prognostic values. In both OPSCC and HPSCC, the IHC results revealed that positive staining for HPV16 E6 protein in tumour samples was associated with better survival rates. However, it was observed that high expression of either p16 or p53 often coincided with E6, which could be considered a positive outcome marker for patients. Additionally, there is a possibility that the E6 oncogene may not have had sufficient time to disrupt the cell cycle. For instance, E6 initiates proteasome-dependent degradation of p53 by recruiting the ubiquitin ligase E6AP. Moreover, only the combined complex of E6 and E6AP can interact with p53. This implies that the expression of a single HPV16 E6 protein may not affect p53 degradation, making its detection less informative for predicting patient outcomes (Li et al., 2019). Unfortunately, this research did not investigate E6AP activity. However, in patients with HPSCC, E6 protein was detected in IHC,

while E6 mRNA was not detected, and HPV16 DNA remained detectable. This finding may indirectly indicate the presence of a persistent HPV16 infection, which could be one of the reasons why the presence of HPV16 DNA in HPSCC samples was associated with worse outcomes.

E7 is considered the major transforming protein of HR-HPVs based on mutational analyses (Basukala & Banks, 2021). Moreover, E7 has been shown to play a crucial role in driving early tumorigenesis (Song et al., 2000). The current study demonstrates that the overexpression of HPV16 E7 protein in OPSCC is associated with a poorer prognosis in Cox regression analysis. However, in HPV-associated tumours, the E7 protein is expected to be the driving factor behind p16 overexpression, which is associated with better survival. On the other hand, some studies indicate that p16 overexpression consistently correlates with a favourable response to therapy and better clinical outcomes in OPSCC, and not all cases of p16 overexpression can be attributed to HPV's oncogenic activity (Rich et al., 2009; Fischer et al., 2010). This suggests the existence of additional mechanisms in E7-protein-associated carcinogenesis. Several studies have demonstrated that E7 induces the upregulation of various matrix metalloproteinases (Menges et al., 2006; Srivastava et al., 2015), which have been linked to the promotion of tumour invasiveness (Basukala et al., 2019). Additionally, the protein function of HR-HPV E7 has been associated with a more stable mitotic function necessary for viral genome maintenance and replication (Yu & Munger, 2013). These processes could contribute to an invasive and potentially metastatic cancer phenotype, thereby explaining the poorer prognosis observed in OPSCC with IHC HPV16 E7 protein overexpression (Basukala & Banks, 2021). Oton-Gonzalez et al. found that OPSCC patients with detectable HPV16 E7 protein in their serum had worse relapse-free survival and overall survival. The authors also identified a correlation between E7 protein levels in serum and E7 mRNA expression, leading them to conclude that the source of E7 protein must have been HPV16-

positive cancer, particularly circulating tumour cells, indicating a metastatic process (Oton-Gonzalez et al., 2021). It is important to note that not all tumours are HPV-related, and it has been demonstrated that virus-induced oncogenesis takes a long time to develop, and some patients with HNSCC can have concomitant HPV infections (Basukala & Banks, 2021).

One of the limitations of the present study is the relatively small sample size for each region (oropharynx, larynx, and hypopharynx), which may result in insufficient statistical power and limit the conclusions, particularly for markers that did not reach statistical significance. However, it is difficult to disregard the observed trends of the examined markers and their impact on survival. Another limitation is that nearly all HPSCC samples were FFPE, which could potentially lead to genetic material degradation, particularly RNA. Nonetheless, all samples were suitable for analysis based on the kits' intrinsic control for mRNA detection or the detection of the β-globin gene for DNA quality assessment.

Conclusions

1. Patients with smaller primary tumours, no locoregional lymph node involvement, absence of tobacco and alcohol use, and those who underwent surgical intervention as part of their treatment approach, demonstrated improved OS and DSS, along with lower hazard ratios.
2. HPV infection has a notable impact on the development of HNSCC, particularly in the case of OPSCC. Not only HR-HPVs but also LR-HPVs could affect the survival of the patients with LSCC and OPSCC.
3. Real-time PCR assays amplifying smaller DNA fragments are good and reliable for detecting HPV genetic material in FFPE samples.
4. There is a high prevalence of the HPV16 genotype not only in oropharyngeal but also in laryngeal and hypopharyngeal cancers confirmed by HPV PCR assays.
5. A moderate correlation between detected E6/E7 mRNAs and HPV16 viral load was confirmed in OPSCC, while this correlation was not observed in non-oropharyngeal cancers.
6. The lack of HPV E6/E7 oncoproteins in HPV DNA-positive tumours implies the involvement of alternative tumorigenesis mechanisms distinct from viral integration.
7. p16 overexpression is linked to improved survival outcomes and lower hazard ratios, not only in patients with OPSCC but also in those with HPSCC. The utilization of p53 expression as a prognostic indicator for patients with HNSCC remains a subject of ongoing debate and uncertainty. The evaluation of HPV16 E6 protein expression through IHC represents a valuable prognostic indicator for, both, OPSCC and HPSCC.

List of publications and reports on topics of Doctoral Thesis

Publications

1. Lifsics, A., Rate, E., Tārs, J., Murovska, M., & Groma, V. (2019). Smoking and alcohol abuse – predictive factors in oropharyngeal squamous cell carcinoma: A retrospective study. SHS Web of Conferences, 68 02013. https://doi.org/10.1051/shsconf/20196802013.

2. Lifsics, A., Rate, E., Ivanova, A., Tars, J., Murovska, M., & Groma, V. (2020). Survival analysis of oropharyngeal squamous cell carcinoma patients linked to histopathology, disease stage, tumor stage, risk factors, and received therapy. Experimental oncology, 42(1), 51–59. https://doi.org/10.32471/exp-oncology.2312-8852.vol-42-no-1.14147.

3. Lifšics, A., Čistjakovs, M., Groma, V. & Murovska, M. (2021). Detection and Genotyping of Human Papillomavirus in Hypopharyngeal Carcinoma Samples. Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences.,75(1), 11-15. https://doi.org/10.2478/prolas-2021-0002.

4. Lifsics, A., Groma, V., Cistjakovs, M., Skuja, S., Deksnis, R., & Murovska, M. (2021). Identification of High-Risk Human Papillomavirus DNA, p16, and E6/E7 Oncoproteins in Laryngeal and Hypopharyngeal Squamous Cell Carcinomas. Viruses, 13(6), 1008. https://doi.org/10.3390/v13061008

5. Lifsics, A., Cistjakovs, M., Sokolovska, L., Deksnis, R., Murovska, M., & Groma, V. (2023). The Role of the p16 and p53 Tumor Suppressor Proteins and Viral HPV16 E6 and E7 Oncoproteins in the Assessment of Survival in Patients with Head and Neck Cancers Associated with Human Papillomavirus Infections. Cancers, 15(10), 2722. https://doi.org/10.3390/cancers15102722

Reports and theses at international congresses and conferences

1. Lifšics, A., Tārs, J., Ivanova, A., Safronovs, J., Groma, V., Murovska, M. Survival rates of patients with oropharyngeal squamous cell carcinoma at Riga East Clinical University Hospital. 2016.gada Zinātniskās konferences tēzes (Rīga, 2016. g. 17.-18. martā) / Rīgas Stradiņa universitāte. Rīga, 2016. 214. lpp. (Poster presentation)

2. Lifšics, A., Veinberga, L., Groma, V., Rāte, E., and Murovska, M. The prevalence of high-risk HPV in patients with oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinomas. RSU Scientific Conference (Rīga, 22–23 March 2018): Abstracts. Rīga: RSU, IX p., 98 p. (Poster presentation)

3. Sokolovska, L., Čistjakovs, M., Sultanova, A., Lifšics, A., Čapenko, S., and Murovska, M. Detection of High-risk Human Papillomaviruses Type Frequency and Viral Load in Latvian Patients with Laryngeal/Oropharyngeal Cancer. RSU Scientific Conference (Rīga, 22–23 March 2018): Abstracts. Rīga: RSU, IV p., 39 p. (Oral presentation)

4. Lifšics, A., Groma, V., and Murovska, M. 2018. Smoking and alcohol abuse – predictive factors in oropharyngeal squamous cell carcinoma (a retrospective

study). 7th International Interdisciplinary Scientific Conference "Society. Health. Welfare: Contemporary Social Dynamics and Welfare: Urban and Rural Development Perspectives" (Rīga, 10-12 October 2018): Abstracts. Rīga: RSU, P.79. (Oral presentation)

5. Lifsics, A., Cistjakovs, M., Groma, V., Murovska, M. Detection and genotyping of human papillomavirus in hypopharyngeal carcinoma samples. Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice" (Rīga, 24–26 March 2021): Abstracts. Rīga: RSU, xix p., 249 p. (Oral presentation)

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Annexes

Immunohistochemical evaluation of HNSCC samples

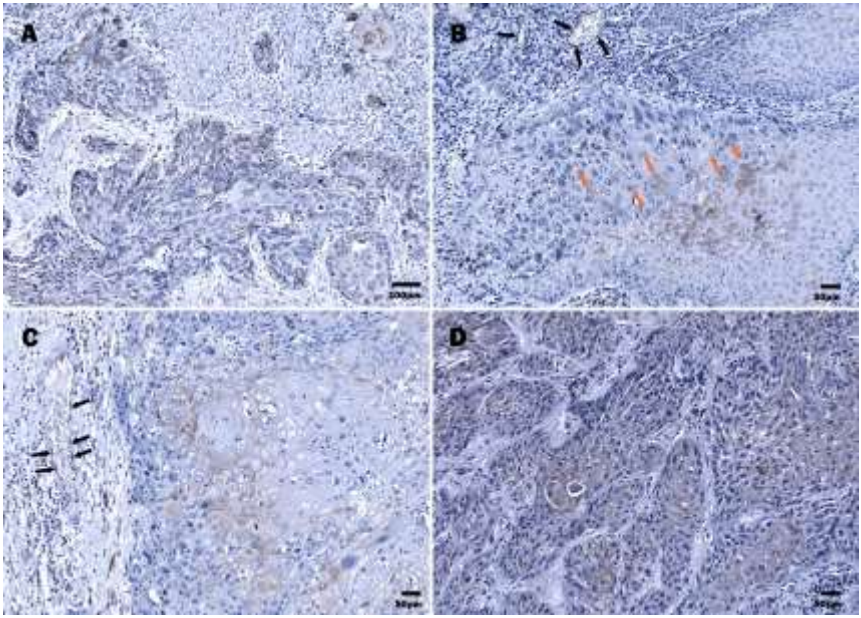

Figure 1 **IHC detection of HPV16 E6 oncoprotein***

* (**A**) LSCC, tumour cords and nests comprised of diffusely distributed E6 protein-positive cells interspersed by the E6 oncoprotein-negative cells; (**B**) LSCC, differentiated suprabasal tumour cells demonstrating abundant HPV16 E6-positive cytoplasm and polymorphous nuclei (orange arrows), E6-positive endotheliocytes (black arrows) within a tumour stroma; (**C**) LSCC, HPV16 E6 positivity in suprabasal, more differentiated, tumour cells, E6-positive endothelial cells (black arrows); (**D**) HPSCC, densely packed tumour cords demonstrating HPV16 E6 oncoprotein positivity, almost exclusively in more differentiated cells.

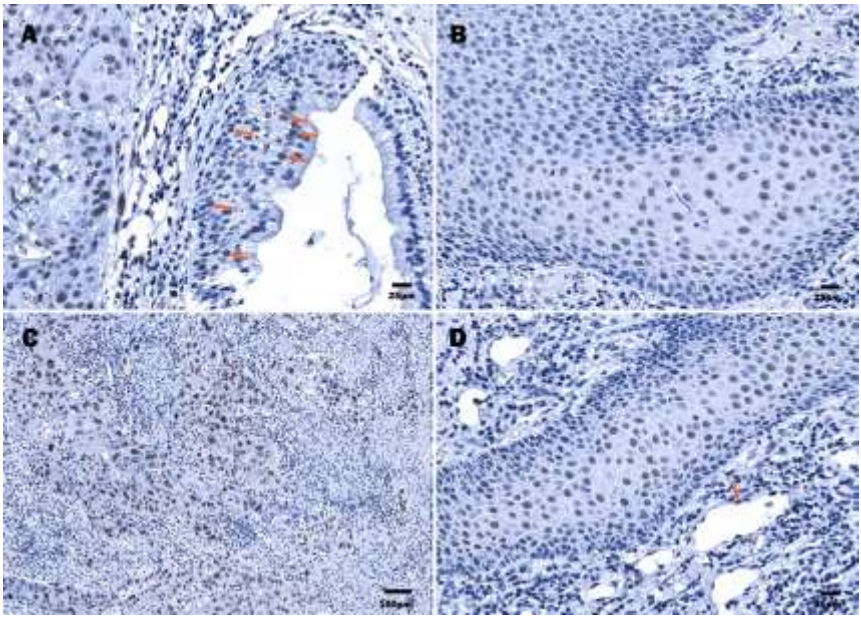

Figure 2 **IHC detection of HPV16 E7 oncoprotein***

* (**A**) LSCC, tumour cells within a nest and some surface cells (orange arrows) demonstrating nuclear HPV16 E7 positivity; (**B**) LSCC, numerous HPV16 E7-positive cells displaying nuclear immunostaining pattern; (**C**) LSCC, highly polymorphous HPV16 E7-positive tumour cells demonstrating nearly total nuclear decoration; (**D**) HPSCC, numerous HPV16 E7-positive cells displaying nuclear immunostaining pattern, endothelial (orange arrow) cells.

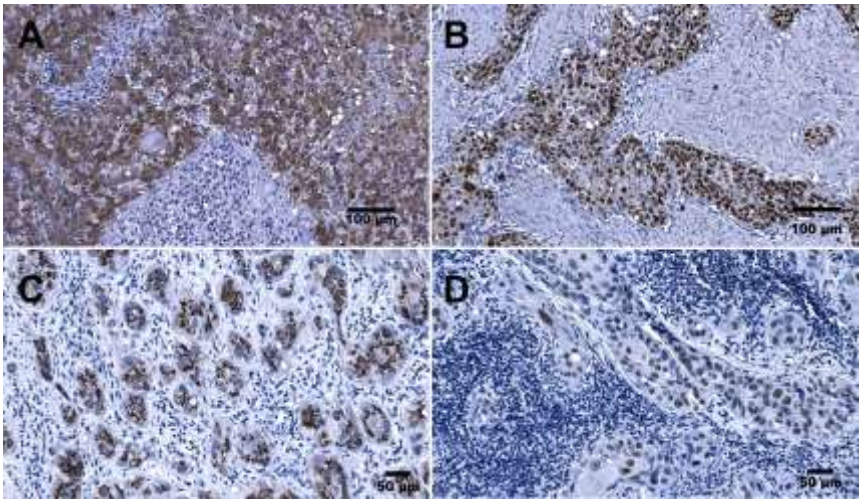

Figure 3 **IHC detection of p16, p53, HPV16 E6 and E7 antigens in HNSCC***

* (**A**) OPSCC (palatine tonsil). Representative image from a case demonstrating > 75 % p16-positive tumour cells displaying mostly nuclear and cytoplasmic expression. (**B**) LSCC. Representative image of p53 overexpression demonstrating uniform strong nuclear staining of tumour cells. (**C**) OPSCC (palatine tonsil). Representative image demonstrating cytoplasmic expression of HPV16 E6 protein confirmed in tumour cells organised in cords. (**D**) OPSCC (palatine tonsil). Representative image demonstrating nuclear expression of HPV16 E7 protein confirmed in the tumour cells organised as nests and cords. Scale bars: 100 µm and 50 µm.

Statistical analysis

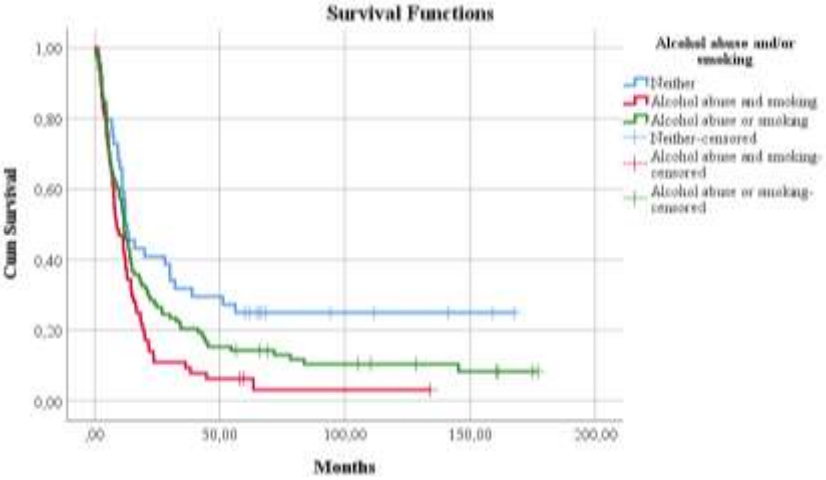

Figure 1 **Kaplan-Meier DSS plot according to hazards**

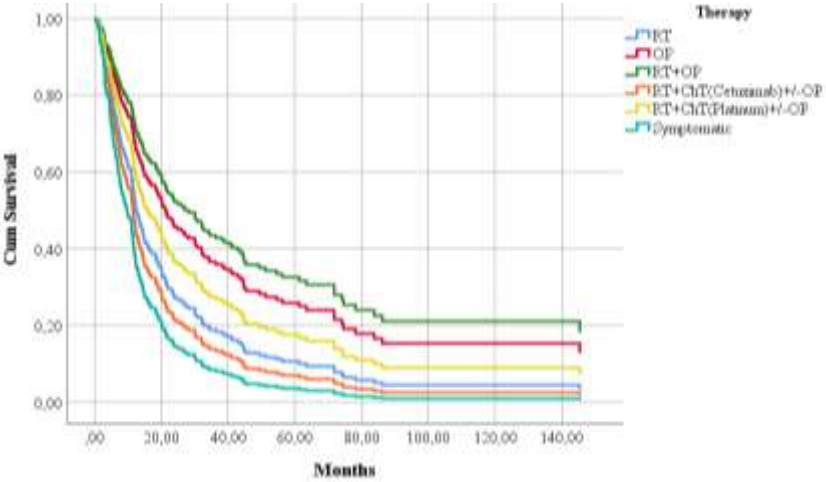

Figure 2 **Cox regression plot for cumulative survival***

* Covariates – sex, age group, T stage, N status, alcohol abuse and/or smoking, therapy, primary tumour location, histological variant. Plot for therapy.

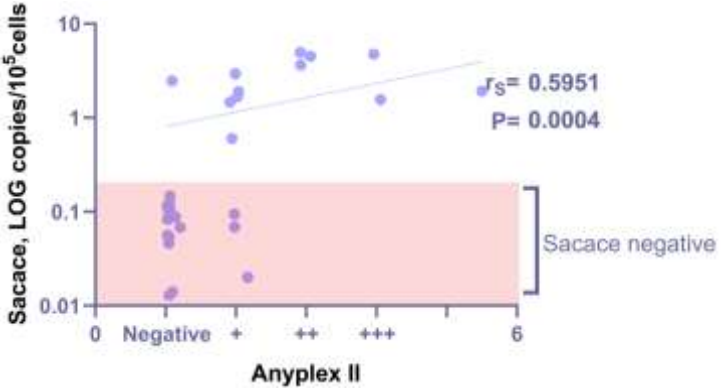

Figure 3 **Correlation of two real-time PCR assays**

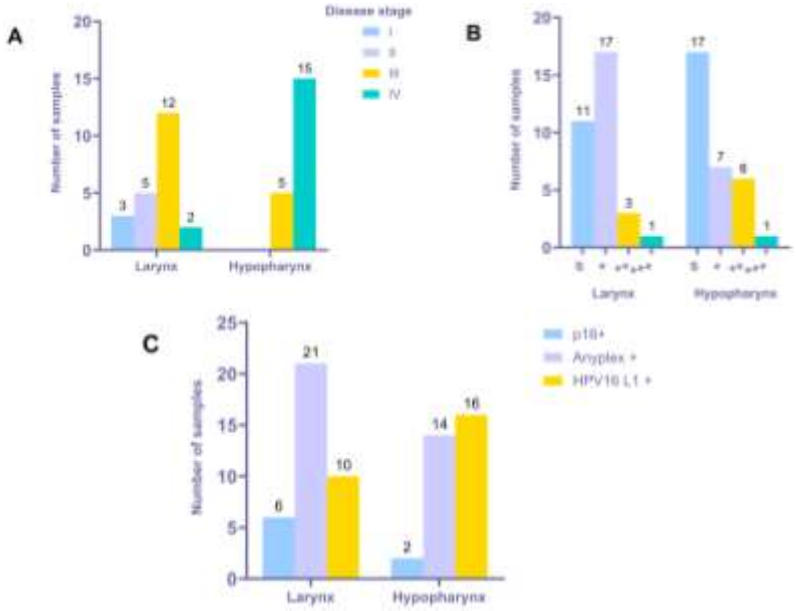

Figure 4 **Distribution of HPV16+ tumour samples according to location, disease stage, and PCR data***

* (**A**) Distribution of HPV16+ tumour samples according to location and disease stage. (**B**) Distribution of HPV16+ samples according to location and Anyplex assay results; 0 – negative, + low viral load, ++ moderate viral load, +++ high viral load. (**C**) Distribution of HPV16+ samples according to location, p16 IHC and genotyping results.

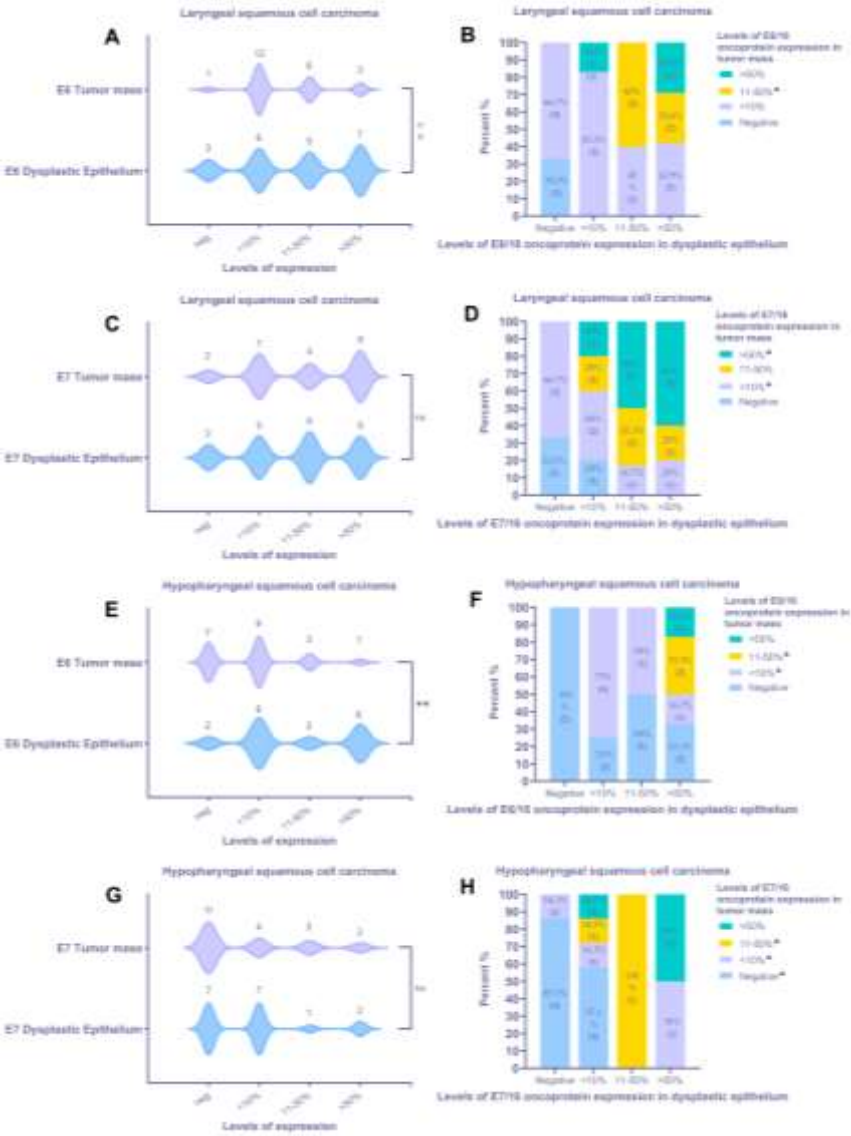

Figure 5 **Assessment of viral oncoproteins E6 and E7 in HPV16+ laryngeal (a-d) and hypopharyngeal (e-h) tumour tissue samples using IHC and statistics***

* (**A, C**) Characterization of HPV oncoprotein E6 (**A**) and E7 (**C**) immunoexpression within a tumour mass and dysplastic epithelium of LSCC samples; (**B, D**) The IHC expression levels for HPV oncoprotein E6 (**B**) and E7 (**D**) in a tumour mass assessed in relation to the levels in a dysplastic epithelium of the corresponding LSCC sample; (**E, G**) Characterization of HPV oncoprotein E6 (**E**) and E7 (**G**) immunoexpression within a tumour mass and dysplastic epithelium of HPSCC samples; (**F, H**) The IHC expression levels for HPV oncoprotein E6 (**F**) and E7 (**H**) in a tumour mass assessed in relation to the levels in a dysplastic epithelium of the corresponding HPSCC sample; Violin plots: asterisks represent a significance level (ns – non-significant, * $p < 0.05$, ** $p < 0.01$) of differences between groups (two-tailed Wilcoxon test); Stacked bar graphs - crosstab analysis, triangles (▲) represent a sample lacking epithelial region suitable for assessment and, therefore, excluded from crosstab analysis.

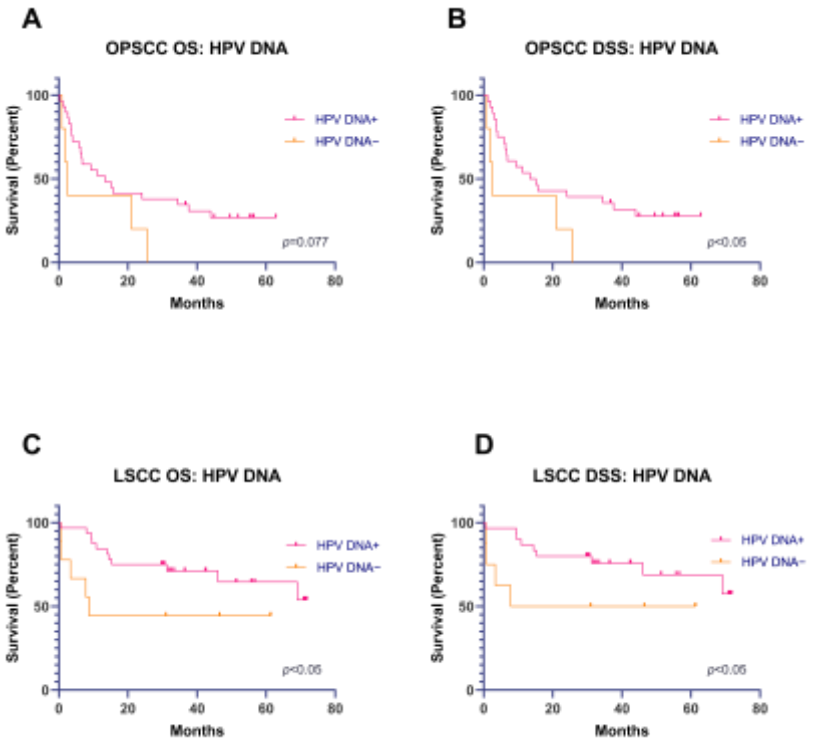

Figure 6 **Kaplan-Meier survival analyses***

* (**A, B**) OS and DSS estimates depending on the presence of HPV DNA (HR- and LR-) in OPSCC; (**C, D**) OS and DSS estimates depending on the presence of HPV DNA (HR- and LR-) in LSCC.

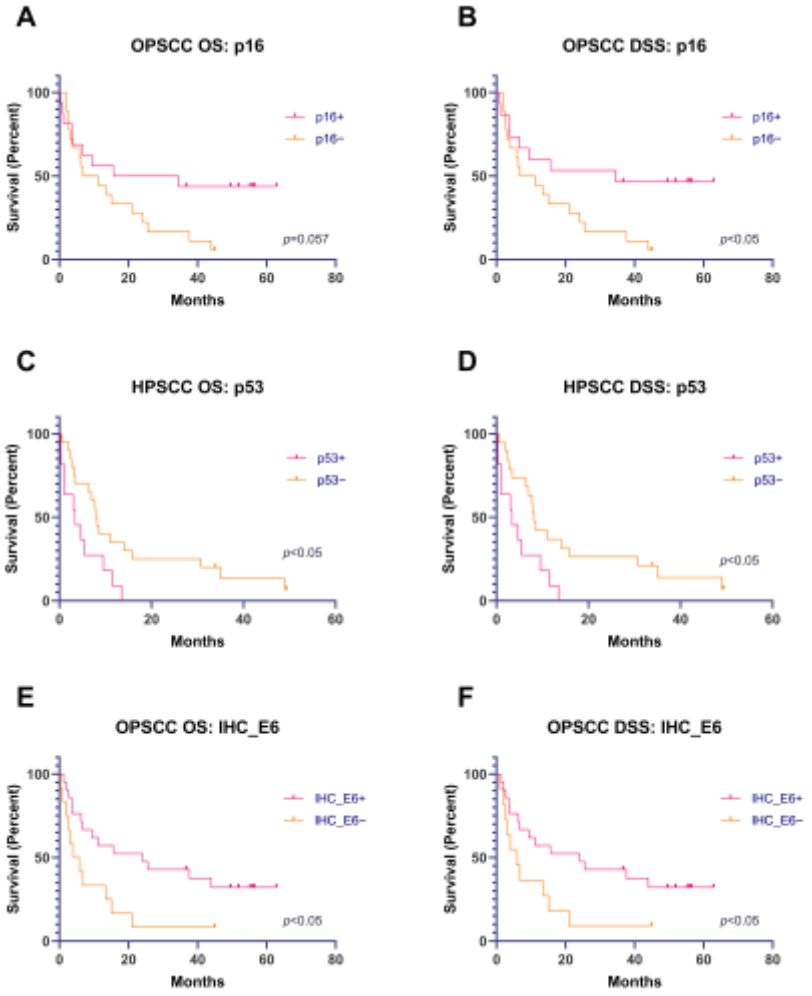

Figure 7 **Kaplan – Meier survival analyses***

* (**A, B**) OS and DSS estimates depending on the result of the IHC expression of p16 in OPSCC; (**C, D**) OS and DSS estimates depending on the results of the IHC expression of p53 in HPSCC; (**E, F**) OS and DSS estimates depending on the results of the IHC expression of HPV16 E6 protein in OPSCC.

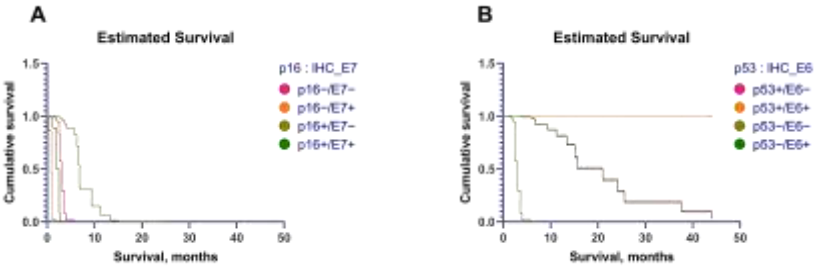

Figure 8 **Survival estimates, Cox regression***

* (**A**) Estimated survival, depending on the IHC expression of p16 and E7 protein; (**B**) Estimated survival, depending on the IHC expression of p53 and E6 protein.

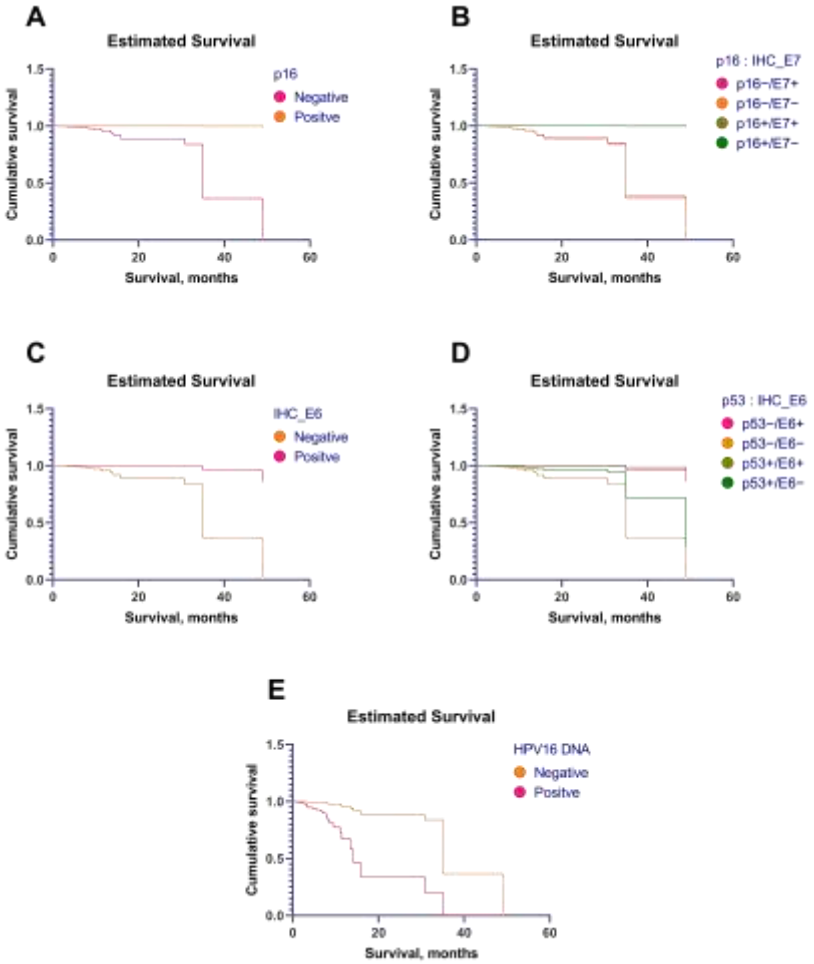

Figure 9 **Survival estimates, Cox regression***

* (**A**) Estimated survival depending on the IHC expression of p16;
(**B**) Estimated survival depending on the IHC expression of p16 and HPV16 E7 protein.
(**C**) Estimated survival depending on the IHC expression of HPV16 E6 protein;
(**D**) Estimated survival depending on the IHC expression of p53 and HPV16 E6 protein;
(**E**) Estimated survival depending on the presence of HPV16 DNA.